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AFLATOXIN ELIMINATION WORKSHOP

St. Louis, MO
October 25-27, 1998

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Throughout the world, aflatoxin is considered one of the most serious food safety problems. Chronic problems with aflatoxin contamination occur in the southern U.S. cottonseed, corn, peanuts and tree nuts. However, the impact of aflatoxin contamination on the agricultural economy is especially devastating during drought years when aflatoxin affects the Midwestern corn belt. Estimated economic losses in years of major aflatoxin outbreaks have been in the hundreds of millions of dollars.

The Aflatoxin Elimination Workshops have served to bring together the Agricultural Research Service (ARS, USDA), university scientists, and representatives of the cottonseed, corn, peanut, and tree nut industries in a unique cooperative effort to develop aflatoxin control strategies through research and development. The ultimate goal of this effort is to facilitate the commercial implementation of technologies to eliminate the aflatoxin contamination problem in the U.S. marketplace by the turn of the millennium. Most of the research to eliminate aflatoxin is conducted by ARS; however, an important addition to this effort is a competitive award program provided by Congressional appropriations to bring to the research effort the talents of university scientists in cooperation with ARS.

Scientists realized over a decade ago that the aflatoxin problem could not be solved solely by conventional technologies utilized routinely to control the more "typical" plant pathogens. Aflatoxin contamination is a result of fungal infection of host plants by a unique class of microorganisms adapted to subsist saprophytically on organic debris in the field or to infect and produce aflatoxin in living plant tissues. Classical plant disease prevention methods developed to control very fastidious plant pathogens have been generally unsuccessful in excluding aflatoxin producing fungi from their relatively broad ecological niche. The realization of the unique nature of the aflatoxin problem and that novel technologies will be required for its control became a focal point of discussion during strategy development sessions of the first Aflatoxin Elimination Workshop in 1988. Two areas of research and development based on the biology and ecology of *Aspergillus flavus*-group fungi were suggested: (1) novel genetic engineering and/or marker-based breeding methods to enhance general antifungal resistance in crops, and
(2) the isolation and formulation of special fungi for use in biocontrol. These biocontrol fungi are strains of *A flavus*-group fungi that do not produce aflatoxin, but have the capability to occupy the same ecological niche in the field and out compete harmful toxin-producing fungi.

The vision of participants in the first Aflatoxin Elimination Workshop has been confirmed by the rapid progress reported in subsequent workshops in developing practical, commercially viable aflatoxin control procedures, based almost entirely on the concepts generally established during the first workshop. Studies on the inheritance of aflatoxin resistance in corn have shown the importance of additive gene action, and chromosome regions associated with resistance have been identified through marker assisted gene mapping. Marker assisted selection is being used to combine genes for insect resistance (e.g., maysin concentration in silks) from one corn line with genes for resistance to *A. flavus* from a second corn line. Recent work has shown that antifungal chitinases are important

resistance factors in the corn line Tex6. A green fluorescent protein (GFP) "reporter" construct represents a valuable new tool for characterizing mechanisms of resistance to *A. flavus* infection.

Improving crop resistance to insects through genetic engineering offers an indirect approach for reducing the incidence of damaged, molded, and aflatoxin-contaminated seeds. Transgenic lines of peanuts harboring the cryIAc gene from *Bacillus thurengiensis* showed a clear reduction in foliar damage by the lesser corn borer in field tests, while walnut tissues harboring this gene caused substantial mortality to Codling moth with an *in vitro* feeding assay. Likewise, some initial transformants of corn, expressing tobacco anionic peroxidase in the leaves, killed 100% of European corn borers and corn earworms, and significantly reduced growth rates of fall armyworms. Field experiments in Illlinois have shown that a substantial reduction in symptoms of visible kernel-ear rot by mycotoxigenic fungi and mycotoxins can occur when the target insect (European corn borer) is present, even at high numbers. Newer Bt genes that produce proteins relatively more toxic to corn earworms should help lower corn earworm incidence and associated mycotoxigenic molds and mycotoxins.

Successful control of aflatoxin in Arizona cotton through the application of a naturally-occurring atoxigenic strain of *A. flavus* was demonstrated by large-scale testing under EPA Experimental Use Permit, and application has been made for full EPA registration. The Arizona Cotton Research & Protection Council is building a facility to produce enough biocontrol inoculum by the year 2000 to treat 200,000 acres of cotton. Similarly, atoxigenic strains of *A. flavus* are being developed to displace and control naturally occurring aflatoxin producing strains in peanut.

Although biologically-based technologies are important themes of the workshops, improvement of crop management and handling practices has also been emphasized. Expanded alogrithms for a newly developed image sorter can recognize certain shell characteristics in pistachios that are characteristic for nuts that are associated with aflatoxin contamination. Careful crop management/handling practices will probably always be important even with the use of powerful new biologically-based technologies.

Research information summarized in this 1998 workshop proceedings provides the technological foundation for the multiple strategies currently being investigated to eliminate aflatoxin, thus leading to the well grounded optimism that solutions to this serious food safety problem will be available by the beginning of the next century. This innovative aflatoxin control technology under development was made possible only by the ingenious application of research information on the nature of *A. flavus*-group fungi and of the aflatoxin contamination process, knowledge which has been provided over the years by scientists attending the workshop.

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CROP RESISTANCE - CONVENTIONAL BREEDING

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Panel Discussion Title: Enhancing Crop Resistance by Conventional Breeding.

Panel Members: Corley Holbrook (Chair), Robert Brown, Thomas Gradziel, Baozhu Guo, Charles Martinson, Torbert R. Rocheford, Don White, Neil Widstrom, and Dave Wilson.

Summary of Presentations: Steady progress continues to be made in enhancing crop resistance to aflatoxin contamination by conventional breeding. Based on the research results presented in this session, it appears clear the conventional breeding will play a vital role in reducing aflatoxin contamination in maize, peanut, and almond.

Don White reported on his group's success in identification and use of sources of resistance in corn. The best sources of resistance are inbred lines Tex6, CI2, and MI82, and most of this group's efforts have been devoted to research with Tex6. Studies on the inheritance of the resistance in Tex6 have shown high broad sense heritabilities, and analysis of generation means indicate that additive gene action is of primary importance for resistance in the crosses studied. Torbert Rocheford reported on his work on mapping genes for resistance in these crosses. He has identified chromosome regions associated with resistance. These may prove useful in marker assisted selection, however, there is still a need for greater probe density in order to more highly saturate the regions of resistance. This group has made considerable progress in transferring resistance into commercially used inbreds while maintaining yield.

Baozhu Guo reported on work involving combining genes for resistance to *Aspergillus flavus* with genes for resistance to corn earworm. The genes for fungal resistance originated from the GT-MAS:gk population, and the genes for insect resistance originated from lines having high maysin concentration in silks. DNA markers associated with maysin concentration have been identified, and this group is looking for QTLs associated with resistance to *A. flavus*. This will enable the transfer of genes for insect resistance and aflatoxin resistance into elite corn inbreds using a backcross breeding program with marker-assisted selection.

Robert Brown reported on research to evaluate GUS and green fluorescent protein (GFP) reporter constructs as tools for examining resistance to preharvest aflatoxin contamination in maize. Both show potential as powerful tools to define or characterize mechanisms of resistance to fungal infection. Additional research showed that maize kernel trypsin inhibitor (TI) is effective in reducing growth of *Aspergillus*.

Charles Martinson described his research on mutagenized B73 and A632 inbred lines of maize. Initial selection was based on a bioassay for inhibitors of aflatoxin synthesis in hexane extracts of maize seed. Subsequent field studies have demonstrated that the selected mutagenized lines are less susceptible to aflatoxin accumulation.

Neil Widstorm focused on the use of a NOR mutant as an inexpensive method for initial screening of corn genotypes for resistance to *A. flavus* infection and aflatoxin contamination. He concluded that this mutant can be used in initial screening to eliminate very susceptible material. This should result in substantial cost savings by eliminating the numerous aflatoxin analyses previously needed for identification of resistance germplasm in mass-screening programs.

Dave Wilson also discussed aspects of the use of the NOR mutant for screening corn and peanut. He agreed that this mutant could be a useful screening tool in corn. However, the use of NOR in drought stressed peanuts was not effective in tests in Tifton because very few red kernels were observed. In addition, results were presented on the development of inoculation procedures using *A. flavus* and *A. parasiticus* for screening peanut for resistance in Georgia and Arizona.

Thomas Gradziel discussed research on the development of resistance to preharvest aflatoxin contamination in almond. An integrated resistance to preharvest aflatoxin contamination is being pursued through the development of resistance to navel orangeworm primarily through high levels of shell integrity, but also through hull and kernel antibiosis/nonpreference, and through the development of resistance of *A. flavus* in the kernel and seed coat.

Corley Holbrook presented results from studies examining three agronomic that had been reported to have the potential to reduce aflatoxin contamination in peanut. These included 1) planting in twin rows, 2) use of a broad spectrum fungicide, 3) use of growth regulator. None of these practices were observed to have a significant impact on aflatoxin contamination. Data were also presented on the most aflatoxin resistant accessions from the peanut core collection. Six accessions have exhibited a 90% reduction in aflatoxin contamination through multiple years of testing.

Summary of Panel Discussion: Don Wicklow ask about the ability of the NOR mutant to suppress the wild type fungi and the ability of the NOR mutant to produce aflatoxin. Dave Wilson answered that the NOR mutant does produce high amounts of aflatoxin in addition to norslorinic acid.

Haixin Xu asked if there were any distinct morphological features that can be used to separated toxigenic from nontoxigenic *Aspergillus*? Dave Wilson replied that to his knowledge there were no distinct morphological differences between toxigenic and nontoxigenic *Aspergillus*.

Joe Dorner asked about the use of early maturity peanuts to possibly escape late season drought stress and subsequent aflatoxin contamination. Corley Holbrook agreed that this may be an attractive approach if the early maturing material was a Runner type of peanut. Unfortunately, most of the early maturing material in the U.S. germplasm collection is Valencia or Spanish type peanut, which is not desirable for most U.S. breeding programs.

Charles Martinson was asked how he produced his corn mutants, and whether efforts were underway to identify and clone the mutated genes. He responded that these mutants were produced using ethyl methane sulfonate (EMS), and that efforts are underway to identify the compounds responsible for the resistance, which have been determined to be hexane soluble.

Thomas Gradziel was asked to expound on the possible causes of his observation that increased nitrogen fertilization rates increased susceptibility. He responded that the major effect was a reduction in shell integrity. In general, conditions which encourage rapid fruit growth during endocarp lignification appear to result in inconsistencies in final shell structure, and thus greater vulnerability to worms.

In answer to a question as why his studies included a growth regulator, Corley Holbrook responded that this growth regulator had been reported to enhance drought tolerance of peanut. However, his group saw no effect on yield under drought stress conditions, and no effect on aflatoxin contamination.

Don White was asked about the origin of Tex6. and responded that Tex6 was a line that he selfed out of an open pollinated variety that was a plant introduction called white master. It is resistant to southern corn leaf blight and has white kernels and white cobs.

In answer to a question from Don White, Charles Martinson replied that EMS corn mutants were morphologically similar to B73 and A632, and that their yield was similar to the parent material.

During panel discussions on possible mechanisms of resistance Robert Brown stated that the proteins in his studies are antifungal, and inhibit the growth of the fungus. However, the ultimate bottom line for germplasm screening is aflatoxin contamination and not fungal growth. unless that is correlated with contamination levels. His group has observed a positive association between fungal growth and aflatoxin contamination levels, and he believes that antifungal proteins such as trypsin inhibitors are an important part of the observed resistance against aflatoxin accumulation. His goal is to combine this type of resistance with factors that provide resistance to aflatoxin biosynthesis. Dave Wilson stated that there seems to be three different kinds of resistance: one is to the fungus, and by virtue of that to aflatoxin accumulation, one is to aflatoxin biosynthesis alone, and the other is a combination of these two.

Dave Wilson expressed his concern that there has not been sufficient effort devoted to bioassay methods. that good methods are needed to differentiate the possible mechanisms of resistance to aflatoxin contamination, and that additional efforts are needed to develop improved methods for chemical analysis of aflatoxin. This is particularly important for breeding programs that are dealing with large sample numbers. Howard Valentine responded from the audience that the work of Tom Whitaker indicated that the problem, at least in commercial peanut lots, is not so much an assay problem, as it is a sampling problem. It is very difficult to obtain samples of commodities for measurement that are representative of the population.

PLATFORM PRESENTATIONS

INHERITANCE OF, MOLECULAR MARKERS ASSOCIATED WITH, AND BREEDING FOR, RESISTANCE TO *ASPERGILLUS* EAR ROT AND AFLATOXIN PRODUCTION IN CORN USING TEX6

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Research at the University of Illinois is divided into four interrelated components including: 1) identifying sources of resistance; 2) determining the inheritance of resistance; 3) molecular marker mapping of genes for resistance; and 4) crossing resistance into commercially used B73 and/or Mo17 type inbreds.

Identification of Resistance. We have screened more than 1,200 corn inbreds as F1 crosses with the susceptible inbreds Mo17 and/or B73 for resistance to Aspergillus ear rot and aflatoxin production. All screening has been done using artificial inoculation. We have identified 13 inbreds that are highly resistant in F1 combinations and as inbreds per se. Many of the 13 inbreds have been shown to have resistance in field or laboratory studies done by others. Our best sources of resistance are inbred lines Tex6, CI2, and MI82. We have devoted most of our efforts the last several years to Tex6. This line was selfed from a white corn variety (PI401762) that had been grown in the southern US.

Inheritance of Resistance. Inheritance of resistance in the inbred Tex 6 has been studied in crosses with susceptible inbreds Mo17 and B73 following inoculation in the field. From 1994 until the present the plant generations included in our studies are the susceptible parent (P_1), the resistant parent (P_2), F_1, F_2, F_3, BCP_1, BCP_1-selfed, and BCP_2. The BCP_2-selfed generation was added in 1996 and 1997 for the B73 X Tex6 cross. In general, F_1 means deviated from the mid-parent value for resistance to aflatoxin production in both crosses indicating dominance for resistance. Analysis of generation means indicate that additive gene action is of primary importance for resistance to aflatoxin production in both crosses. Broad sense heritabilities are relatively high in crosses with both Mo17 and B73. Our biggest problem is that environmental conditions in 1996 were not favorable for aflatoxin production. We have, therefore, repeated the experiments in an attempt to develop the necessary data for our RFLP mapping.

Mapping genes for Resistance. We have completed the molecular marker mapping in the Tex6 x Mo17 F3 mapping population. We are approximately halfway through the mapping in the Tex6 x B73 F3 and BC1 mapping populations (45 marker loci). We have identified chromosomal regions associated with resistance to ear rot and with inhibition of aflatoxin production in all three Tex6 mapping populations. There are a number of general chromosomal regions associated with resistance that are common between the Tex6 x Mo17 and the Tex6 x B73 mapping populations. This provides supportive evidence for the validity of these chromosome regions. We also have identified chromosome regions that are not in common between the different Tex6 mapping populations. This is not surprising because Tex6 and Mo17 may have some of the same genes for resistance in common that Tex6 and B73 do not have in common, and vice versa. This suggests that certain chromosome regions of Tex6 could be used to improve

Mo17 type lines and other Tex6 regions used to improve B73 type lines. Comparison of chromosome regions associated with inhibition of aflatoxin levels across all mapping studies (includes LB31xB73 and R001xB73 F3s) reveals that there are some chromosome regions in common. The regions on chromosome arms 2L, 3L, 4S, and 8S may be the most promising for marker assisted introgression into commercial lines for improving resistance.

Breeding for disease resistance. We've made considerable progress in transferring resistance from the inbred LB 31 and Tex6 into commercially used inbreds while maintaining yield. With LB31 we have crossed resistance into FR1064, which is a widely used B73 type, and FR2128 which is a later maturing B73 type used in the southern US. Both FR1064 and FR2128 have yellow kernels. We are also working with white corn and have crossed resistance from Tex6 into FR808 which is a B73 type and into FR810 which is a Mo17 type. We also have early generations of crosses between Tex6, Mo17, and FR697 which is also an Mo17 type. We can cross resistance into cornbelt related lines, however, we are not recovering the high level of resistance that is seen in Tex6. Presumably we are not transferring all genes for resistance into new individual lines. We have started performing SSR marker assays on the lines in development. We are probing in the regions where associations with resistance have been identified in the mapping studies. This will provide information on whether a specific chromosome region from a resistance source has been introgressed into a more elite line. Since these lines are agronomically superior to the original resistance sources, they are more desirable for use as sources of resistance by the hybrid seed industry. The SSR marker information will make these lines even more useful for marker assisted selection by the hybrid seed industry. Unfortunately, due to the lack of funding, our breeding program has never developed to the size necessary for rapid creation of agronomically superior lines with good levels of resistance.

Conclusions. High levels of resistance have been identified and can be transferred into usable germplasm. In most cases, it appears that resistance is under the control of several genes acting in an additive fashion, even though these genes do confer resistance in F1 crosses. Marker assisted selection is not a reality at this time, however, it has provided valuable information on chromosomal regions associated with resistance.

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PYRAMIDING RESISTANCE TO *ASPERGILLUS FLAVUS* AND CORN EARWORM TO CONTROL PREHARVEST AFLATOXIN CONTAMINATION: GENETIC MAPPING AND DNA MARKERS LINKED TO RESISTANCE TRAITS

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Resistance sources and resistance mechanisms to *A. flavus* infection and to corn earworm damage have been identified. The objective of this research is to control preharvest aflatoxin contamination of corn through pyramiding resistance to ear-feeding insects and resistance to *Aspergillus flavus*. Recently we have identified DNA-markers associated with maysin concentration, an antibiotic factor, in corn silks. Using the same approach, we will identify QTLs associated with resistance to *A. flavus* infection of corn. The ultimate goal is to transfer genes for insect resistance and aflatoxin resistance into elite corn inbreds using a backcross breeding program, and to develop corn inbreds with resistance to ear-feeding insects and aflatoxin production using marker-assisted selection. Using RAPD-PCR, we have shown that there is genetic diversity within the corn population GT-MAS:gk, identified as germplasm resistant to *A. flavus*. The resistance in this population has been stabilized with selfing and GT-A1 and GT-A2 have been selected as the most resistant lines. RAPD and RFLP markers identified may be linked with the resistant traits. We have generated mapping populations for insect resistance and for aflatoxin resistance, separately. $F_{2:3}$ families have been produced for aflatoxin resistance identification. The polymorphic screening among parents and their F1s has been completed using 98 RFLP probes and 6 restriction enzymes. One recessive gene on chromosome 3 has been identified which increased silk maysin production 3-fold in the presence of a functional *p1* locus. The PCR product of this gene (1.4 kb) has been sequenced, and was found to be highly homologous to a transcriptional regulator gene in sorghum. Populations for pyramiding have been generated between aflatoxin resistant lines, GT-A1, GT-A2, and Mp313E, and insect resistant lines, GE37, SC102, and Zaplote Chico. Field evaluation has demonstrated that lines with high silk maysin have reduced corn earworm damage and subsequently reduced aflatoxin production.

MAIZE RESISTANCE TO *ASPERGILLUS FLAVUS* INFECTION: FIELD EVALUATIONS AND BIOCHEMICAL CHARACTERIZATION OF ANTIFUNGAL TRAITS

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Experiments are being conducted using *Aspergillus flavus* transformants expressing β-glucuronidase (GUS) or green fluorescent protein (GFP) as a means of assessing degree of *A.flavus* infection of corn ears and individual kernels. Two resistant (T115, GT-MAS:gk) and two susceptible (Pioneer 3165 and Delta Pine G-4666) maize genotypes were planted in April, 1998. At 12-15 days after midsilk, ears of three genotypes (in T115, there was little to no pollination) were punctured once with a 3mm (diameter) cork borer through husks to wound a kernel(s). A 20 ul spore suspension (4.0×10^6 conidia/ml) of either an *Aspergillus. flavus* transformant expressing GUS or GFP were pipetted through the wound. At maturity, ears were harvested, husked, and stored at 4 C. Each inoculated ear is being assessed for degree of infection through kernels adjacent to wounded kernel(s), and for the degree of spread away from the wound-site. Evaluations are being conducted histochemically and through quantitation of GUS (1, 2) or GFP. Either or both of these reporter constructs may prove useful for evaluating maize genotypes for preharvest resistance to *A. flavus* infection/aflatoxin contamination. They may also enable us to more clearly understand how the fungus spreads and how resistance to fungal infection is expressed.

Seventy six (76) maize inbreds adapted to savanna and midecological zones of Central and West Africa are being screened using the kernel screening assay (KSA) (1), for resistance to aflatoxin production. These lines had been selected for mid to high resistance to ear rot (*Aspergillus. Fusarium. Diplodia. Botrydiplodia*, and *Macropomina*) under severe infection in West Africa. Preliminary screening of nine of the lines along with controls. including a resistant line GT-MAS:gk, and the susceptible line Delta Pine G-4666, indicates significant differences among the African lines with two accumulating aflatoxins at levels nearly as low as the resistant control, GT-MAS:gk. This offers the potential for identifying resistant genotypes among the 76 African inbreds. Resistance mechanisms may be identified in the African lines that differ from those currently being investigated in known resistant genotypes. This would enhance our strategy of pyramiding resistance into selected maize lines to inhibit *A. flavus* infection/aflatoxin contamination.

Several corn kernel biochemical traits being investigated as potential resistance factors, the most fully characterized is the 14 kDa maize kernel trypsin inhibitor (TI). It has been

associated with resistance (3), being expressed in high concentrations in resistant lines but not expressed or expressed in low concentrations in susceptible lines. It also has been shown to inhibit *A. flavus*, *A. parasiticus*, and several other morphologically diverse fungi (4). To determine the mode of action of TI fungal growth inhibition, TI was tested for ability to inhibit *A. flavus* α-amylase. Reports in the literature have described other TI's as inhibitory of α-amylases. Our results indicate that extracellular production of α-amylase in 10% PDB is inhibited over a 24 h period by a 100 ug/ml TI concentration. Interestingly, a 200 ug/ml TI concentration initially inhibits *A. flavus* α-amylase production, however, this is followed by an overproduction of the hydrolase to levels exceeding those of controls. When tested against α-amylase enzymatic activity, TI reduced activity by 27%. No inhibition was demonstrated in glucose or gelatin media. These results indicate that inhibition of α-amylase and thus, fungal ability ability to liberate glucose from starch, may be one of the ways TI inhibits *A. flavus* growth.

Our results have shown that differences exist in constitutive and inducible protein expression between resistant and susceptible maize genotypes. We compared proteins expressed over a 7 day period in kernels of 2 resistant (GT-MAS:gk, Mp420) and 2 susceptible genotypes (Pioneer 3165, Delta Pine G-4666). These mature kernels were not infected with A. flavus. Western blots of these SDS PAGE gels probed with antibodies of known antifungal proteins were performed. Results indicate that antifungal proteins, zeamatin and a 28 kDa chitinase are constituitively expressed in the 2 resistant lines, but are induced over time in the susceptible lines. Ribosome inactivating protein (RIP B inactive form) appears depleted (likely converted to the active form) in GT-MAS:gk by day 3 and is gradually reduced in Mp420 over 7 days. RIP (inactive form) expression remains consistent over 7 days in both susceptible lines. The 14 kDa TI expression is consistent in all 4 lines, however, TI concentration appears higher in resistant lines than in susceptible ones. These results indicate that differences exist in constitutive and inducible protein expression between resistant and susceptible maize genotypes. This may explain findings of a previous study (5) which demonstrated that resistance to aflatoxin accumulation is induced in kernels of susceptible lines that are allowed to imbibe water prior to inoculation. It may also indicate one clear difference between resistant and susceptible kernels: greater expression of constitutive antifungal protein activity in resistant lines to fight initial fungal attack.

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UPDATE ON EFFORTS TO DEVELOP AFLATOXIN RESISTANT B73 AND A632 GERMPLASM

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A total of 8348 M3 families of mutagenized B73 and A632 inbred lines of maize were previously bioassayed for inhibitors of aflatoxin synthesis in hexane extracts of maize seed. Inhibition of aflatoxin synthesis was a rare phenomenon and only six kernels were found. The assay was a destructive test, but through a process of selfing plants grown from residual seed from the same family ear, the frequency of the trait in subsequent families has been concentrated.

In 1997, 20 M6 families of A632 and 40 families of B73 were selected for definitive field trials based on agronomic traits and the fact that each family had at least 70% seeds possessing high levels of inhibition of aflatoxin synthesis. Although extracts from the dry seeds inhibited aflatoxin synthesis, the materials needed to be tested for inhibition of aflatoxin synthesis in the field. Open pollinated ears on the plants in the field were inoculated at the soft dough stage with *Aspergillus flavus* by injection with a syringe and a 14 gauge Hamilton No.5 point style needle directed tangentially through the developing kernels and along the cob. Ears were allowed to mature on the plant. After harvesting the ear and drying at 35 C, the kernels contiguous with the sporulating inoculation site were shelled. Ten kernels that had an intact pericarp and no sign of mummification were surface sterilized for 3 min in 1.3% sodium hypochlorite followed by 1 min in 95% ethyl alcohol. The surface-sterilized kernels were sampled for internal infection by *A. flavus* by boring radially through the embryo and endosperm with a sterile #60 drill bit and plating the core onto PDA. The percentage of sound kernels internally infected were 42% for B73 families and 51-52% for A632 families, regardless of whether or not they had a mutagenized parentage. The kernels containing *A. flavus* were assayed for aflatoxin concentration using a modified Velasco minicolumn procedure that would detect concentrations of aflatoxin B1 above 5 ppb in the kernels. Aflatoxin was detectable in kernels from 20% of the B73 M6 ears at an average concentration of 170 ppb when detectable: by contrast, aflatoxin was detected in 81% of the non-mutagenized normal B73 ears at an average concentration of 346 ppb when detectable. Among the A632 M6 families tested, 19% of the infected ears contained detectable levels of aflatoxin in the infected kernels with an average aflatoxin content of 51 ppb. Among normal A632 ears, 25% of the ears had detectable levels of aflatoxin in the infected kernels with an average concentration of 149 ppb.

The best B73 families in terms of stable effective resistance to aflatoxin synthesis derive from two original M3 seed. In A632 the best families are from one M3 seed.

The better M7 families from each inbred line were selfed for seed increase and crossed to B100 for testing for yield and other agronomic traits in 1999.

The elite mutagenized B73 and A632 germplasm being developed came from the program of Allen D. Wright while he was associated with the USDA-ARS at Iowa State University.

A ROLE FOR THE NOR MUTANT OF *ASPERGILLUS PARASITICUS* IN A BREEDING PROGRAM TO IMPROVE RESISTANCE TO *A. FLAVUS* INFECTION AND AFLATOXIN CONTAMINATION OF CORN

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An effective and inexpensive method is needed for initial screening of corn genotypes for resistance to *A. flavus* infection and aflatoxin contamination. The NOR mutant of *A. parasiticus* produces a reddish stain in the aleurone layer of the corn kernel-when infected with the isolate ATCC24690. Our objective was to determine if this mutant can be useful as a tool to screen maize genotypes for resistance. An experiment with two hybrids over a three-year period demonstrated that effective separation of genotypes may be possible. Another experiment with eight hybrids also gave results that warranted further study. Separation of resistant gentypes among S1 progenies from breeding populations was ineffective, suggesting that the method is not useful for separating genotypes with similar background and resistance levels. However, a randomly chosen group of exotic collections were effectively separated, demonstrating that initial screening of widely differing genotypes can be accomplished using the number of red-stained kernels as an indicator of infection and contamination levels. Initial screening to eliminate the majority of susceptible test genotypes is recommended. The use of this procedure will result in substantial cost savings by eliminating numerous aflatoxin analyses previously needed for identification of resistant germplasm in mass-screening programs.

UTILITY OF AFLATOXIGENIC *ASPERGILLUS FLAVUS* AND *A. PARASITICUS* INOCULUM USED IN SCREENING OF CORN AND PEANUT GERMPLASM FOR RESISTANCE TO AFLATOXIN CONTAMINATION

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Studies on inoculation methods of corn and peanuts with aflatoxigenic *A. flavus* and *A. parasiticus* have been ongoing for many years. The use of mixed inoculum was tried in peanuts in Georgia and Arizona. Both fungi survived and were adequate inoculum sources in Georgia while in Arizona *A. parasiticus* did not survive in the soil and could not be recovered from peanuts in inoculated plots. Therefore only *A. flavus* has been used recently in Arizona while in Georgia a mixed inoculum continues to be used. The advantage of inoculation is that it lowers the variation and helps avoid false negative readings in screening. Both fungi survive quite well in inoculated corn in Georgia and can be used interchangeably with no obvious differences.

The nor *A. parasiticus* mutant has been used in Georgia in corn to study infection and to identify highly susceptible germplasm. The numbers of red kernels stained with nor can be helpful in identifying highly susceptible accessions and in this way minimizes costs. However, the numbers of red kernels vary over years making the technique only useful for initial screening. The use of the nor mutant in drought stressed peanuts was not effective in tests in Tifton, GA because very few red kernels were observed.

The use of the nor mutant is very useful in studying infection of both peanuts and corn because the fungus is easily identified and the metabolite pattern can be followed with HPLC methods. The use of silk inoculation was not effective using the nor mutant but wound inoculation was very effective in establishing high infection percentages and aflatoxin concentrations. The nor mutant competes well in nature and there is a negative correlation between the incidence of the mutant and the wild type *A. flavus* group.

INTEGRATED FUNGAL/INSECT RESISTANCE TO AFLATOXIN CONTAMINATION OF ALMOND APPEARS DURABLE IN FIELD TRIALS DESPITE DIFFERENTIAL ENVIRONMENTAL AND GENOTYPE BY ENVIRONMENT RESPONSES OF INDIVIDUAL COMPONENTS

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An integrated resistance to preharvest aflatoxin contamination is being pursued through the development of resistance to navel orangeworm (NOW) *(Amyelois transitella)* primarily in the form of high levels of endocarp (shell) seal integrity but also through hull and kernel antibiosis/nonpreference and through the development of resistance to *Aspergillus flavus* (AF) in the kernel and seed coat. This strategy is founded on previous research which has shown that the critical period for preharvest aflatoxin contamination occurs during the limited period from hull split to the field drying of almond kernel meats. Aflatoxin contamination occurs when the seed coat, which normally acts as a barrier to infection by AF is damaged usually by feeding by NOW.

NOW feeding studies using standard insect diets supplemented with tissue from either kernels or hulls of the more resistant variety *Mission* and the major commercial variety *Nonpareil* have demonstrated both significantly greater larval mortality as well as significantly longer insect development times when *Mission* tissue was incorporated into the diets. *Mission* was also found to have significantly lower oleic-to- linoleic levels when compared to *Nonpareil*, with the highest levels of this trait being found in lines resulting from gene introgression from wild *Prunus* species. Lines derived from related *Prunus* species also demonstrated some of the lowest levels of aflatoxin B_1 production following controlled inoculations. No relationship was observed between kernel fatty acid composition and either disease or toxin development for over 20 cultivars and genotypes evaluated. A very high correlation was found between oleic and linoleic acid levels (R^2=0.98) suggesting the direct conversion of oleic to linoleic acids by oleic desaturases.

Shell seal integrity was found to be affected by field plot location, rootstock utilized, and levels of nitrogen applied to seed trees. In general, conditions which encourage rapid fruit growth during endocarp lignification appear to result in inconsistencies in final shell structure and so greater vulnerability to worms. Breakdown of shell seal integrity varied with cultivar studied. Resistance to aflatoxin contamination of almond was maintained at high field levels despite the partial breakdown in shell seal integrity observed in some genotypes. The durability of this field resistance is probably due to the integration of multiple layers of resistance in these genotypes and was demonstrated by the absence of appreciable insect infestation and fungal infection of resistant nuts, and by the absence of toxin production in selected genotypes possessing a moderately thick, well sealed shells. While moderately thick shells have previously been associated with low yield potentials due to the high energy costs of lignin production, present studies show no significant relationship between level of lignin production in the shell or endocarp and yield potential.

Genetically engineered almonds are being pursued through the development of efficient transformation and regeneration techniques. Transformation efforts include the use of GFP and CryIAc genes with both the CaMV35S and UBi3 promoters. Regeneration efforts have emphasized the identification of optimal carbohydrate source and balance for the range of genotypes to be engineered.

In-Press

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SEARCHING FOR A SILVER BULLET IN A HAYSTACK

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Preharvest aflatoxin contamination (PAC) is one of the most serious challenges facing the U. S. peanut industry. Since 1990 we have been conducting research to address this challenge. One of the main objectives of this research program has been to identify sources of resistance to PAC and to use these sources to develop resistant peanut cultivars. We have examined germplasm previously reported to have resistance to PAC, germplasm with resistance to other fungi, and germplasm with low linoleic acid. None of this germplasm showed a level of resistance to PAC high enough to be used in a cultivar development program. In 1997 we examined three agronomic practices that had been reported to have the potential to reduce PAC in peanut. These included: 1) planting in twin rather than single rows. 2) The use of abound, a new broad spectrum fungicide, and 3) The use of a new growth regulator. None of these agronomic practices had a significant effect on PAC. We also have recently completed screening of the peanut core collection for sources of resistance to PAC. The core collection is a representative subsample of the entire U.S. germplasm collection. All accession in the core collection were first examined in a preliminary screen using five replications in a single environment. Genotypes that had low aflatoxin contamination levels in the preliminary screen were then retested. Fourteen core accessions were observed to have on average, a 70% reduction in PAC in multiple years of testing. Six of these accessions (99, 147, 276, 282, and 522) exhibited a 90% reduction in PAC in multiple years of testing. The lines that we have identified as having lower PAC have less than acceptable agronomic characteristics. These lines have been entered into a hybridization program to combine resistance to PAC with acceptable agronomic performance. The resulting populations are advanced to the F_4 generation using summer and winter nurseries. Testing within these populations to select high yielding progenies with resistance to PAC began in 1997.

POSTER PRESENTATIONS

GENETIC DIVERSITY IN THE GT-MAS:GK POPULATION AND POSSIBLE PCR AND RFLP MARKERS LINKED TO RESISTANCE TO *ASPERGILLUS FLAVUS*

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Aspergillus flavus infection and subsequent aflatoxin contamination of maize grain is an extremely serious problem. Maize genotypes resistant to *A. flavus* attack are desperately needed. The challenge to maize breeders is to identify resistance sources and to incorporate the improved resistance traits into the breeding material. GT-MAS:gk, identified as resistant germplasm to *A. flavus*, was released for use as a resistance source. The population has been maintained by bulk sibbing without selection and variation in resistance has been observed. In order to utilize the resistance trait in this population, inbreeding with selection for resistance is needed. In the present study, we surveyed the genetic divergence for DNA polymorphism among individual plants from the GT-MAS:gk population using 40 primers. Fifteen primers gave sufficient numbers of reproducible and readily scored polymorphic bands and suggested that this population was moderately homogeneous based on a low frequency (38%) of polymorphic bands with 40 primers. Genetic distance ranging from 0.079 to 0.276 and averaging 0.164 suggests that there is genetic diversity within the population. However, the averaged genetic distance between population GT-MAS:gk and a susceptible sister population GT-MAS:pw.nf, was 0.293. Cluster analysis resulted in three major groupings within the GT-MAS:gk population. Group one has the most resistant individuals with less aflatoxin. Group two most likely has the least resistance, and group three is intermediate. We also tested 11 S4 lines using RFLP markers and clearly demonstrated genetic variation among the 11 lines. The RAPD and RFLP markers potentially linked to resistance are discussed and may be useful for the pyramiding of resistance genes in elite lines and, perhaps, for identifying other germplasm that possesses the resistance genes.

RFLP SCREENING TO MAP RESISTANCE IN CORN TO CORN EARWORM AND *ASPERGILLUS FLAVUS*

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Ear-feeding damage by insects has been consistently associated with increased contamination by aflatoxin. Both types of resistance have been identified in our laboratory; host plant resistance to *Aspergillus flavus* and to corn earworm (*Helicoverpa zea*), a most destructive ear-feeding insect in the southeastern U.S. We have determined that multiple traits influence both types of resistance. The objective of this research is to pyramid both types of resistance to control preharvest aflatoxin contamination of corn (*Zea mays*).

Insect resistance: We constructed QTL mapping populations (F2) and first backcrosses for traits associated with insect resistance, such as maysin concentration in silks, silk channel length, and husk tightness. There are 4 F2 populations (a total of more than 1,500 leaf and silk samples) for insect resistance. We have completed RFLP genotype polymorphism screening using 6 restriction enzymes (*Bam*HI, *Dra*I, *Eco*R1, *Eco*RV, *Hind*III, and *Xba*I) and 98 probes. Preliminary data suggest that a locus on chromosome 3 which increases maysin levels 2-3 times. Selection using RFLP markers on three chromosome regions (*P1* on chromosome 1, *a1-sh2* on chromosome 3, and *bz1-wx1* on chromosome 9) should provide for sufficient maysin in silks for effective antibiotic activity against corn earworm.

Aflatoxin resistance: We generated 6 F2 mapping populations and 4 F2:3 populations for identification of resistance traits to *A. flavus* infection and aflatoxin biosynthesis. Silk maysin concentrations are 0.62, 0.42, and 0.15 (percentage of fresh silk weight) for GT-A1, GT-A2, and Mp313E, respectively, while the silk maysin concentrations in all F1 crosses with low maysin lines are below 0.1%. Gene effects are additive and/or recessive for maysin concentration in these crosses. RFLP polymorphism screening has been completed for these crosses.

Field evaluation and pyramiding: Field experiments demonstrated that it is feasibile to control preharvest aflatoxin contamination through pyramiding resistance to ear-feeding insects and *A. flavus*. Lines with high silk maysin concentration, tight husk and resistance characters to *A. flavus* had less visible insect damage and fungal colonization. Analyses of aflatoxin levels are in progress. We crossed all the parents with an *a1* tester stock. Testcrosses with the *a1* tester revealed that all dent corn parents possess a functional allele (*A1*) while this locus in the *sh2*-type parents is a nonfunctional allele (*a1*). We have initiated pyramiding crosses of high silk maysin and tight husk lines to aflatoxin resistant lines and generated F1s (GT-A1, GT-A2, and Mp313E crossed with 'Zapalote Chico', GE37, and SC102).

CHARACTERIZATION OF A CHROMOSOME REGION WHICH ENHANCES MAYSIN PRODUCTION IN CORN SILKS AND REDUCES EARWORM GROWTH AND EAR DAMAGE

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Aflatoxins, a major group of mycotoxins, are of great concern in food and feed safety worldwide. Ear-feeding damage by insects has been consistently associated with increased contamination by aflatoxin and, as such, is considered a major contributor to the breakdown of natural resistance to *Aspergillus flavus* in corn (*Zea mays*) kernels. We have identified maysin in corn silks, a compound conferring resistance on corn to ear damage by the corn earworm (*Helicoverpa zea*). maize resistance to corn earworm, the most destructive ear-feeding insect, damage to the ear due to maysin in silks. Field evaluations demonstrate the potential for combining insect resistance in maize lines with resistant to *A. flavus* to control preharvest aflatoxin contamination. In F2 mapping populations derived from high maysin dent corn x low maysin sweet corn, we found a recessive maysin enhancer gene in the sweet corn parents, which has a dramatic effect on the concentration of maysin in silks when the enhancer is homozygous. We sequenced the 5' and 3' ends of the plasmid inserts of RFLP probes for *a1* and *sh2* loci using universal primers, M13 forward and reverse, which has 94% and 98% homology to the respective genes. We designed a pair of primers, based on the DNA sequence from rice and sorghum, amplified the genomic DNA of dent corn GE37 and sweet corn B31857, which resulted in a PCR product with 1.4 kb. The completed sequences showed that GE37 lacks the **GTAGG** nucletides which are present in B31857. This sequence is highly homologous to the transcriptional regulator gene in sorghum. Northern analyses showed that there are no differences between mRNA in coleoptiles from dent and sweet corn. Future studies will include mRNA analyses in the silk stage, protein purification, and characterization of the catalytic activity of this protein.

RELIEF OF AFLATOXIN CONTAMINATION THROUGH IMPROVED DROUGHT TOLERANCE

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Previous studies found large rooted peanut plants, peanut leaf temperatures and visual stress ratings were correlated to aflatoxin resistance. Using 20 peanut cultivars from these studies we measured physical characteristics such as leaf temperature and root measurements to try and improve the current selection methods for aflatoxin resistance. Root measurements (total length, surface area, and diameter) were recorded 30 and 60 days after planting, DAP. Drought tolerance was measured by placing rain shelters at 60 DAP over the genotypes and taking infrared leaf temperature and leaf stress measurements twice a week until harvest. Many of the genotypes began to show stress approximately 95 DAP. Those showing the least amount of stress were genotypes 47, 215, 292, 299, and 732; while 158, 287, and 366 exhibited higher during the measurement dates. Root length and root surface area means varied among the genotypes, 2535 mm (genotype 375) - 1150 mm (genotype 299) and 381 mm (genotype 732) - 202 mm (genotype 99), respectively. Root density seem fairly consistent 0.591 mm (genotype 215) - 0.047 mm (genotype 721) throughout the genotypes. Physical characteristics seem to contribute to aflatoxin resistance, but other mechanisms may play a equal or more important role.

RESPONSE OF RESISTANT AND SUSCEPTIBLE MAIZE INBREDS TO INOCULATION WITH TRANSFORMED *ASPERGILLUS FLAVUS* ISOLATES

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Two maize inbred lines resistant to *Aspergillus flavus*, a susceptible line, and their F_1, F_2, and backcross progeny were inoculated in 1997 at 14 and 21 days after pollination with an *A. flavus* strain (GAP 2-4) transformed with the *Escherichia coli* GUS gene linked to β-tubulin promoter to detect fungal colonization of cob and kernel tissues. GUS expression was observed 7 days after inoculation and was highest after 28 days. The expression of GUS activity was concentrated almost exclusively in the embryo tissue, specifically in the scutellum. In the cob tissue expression was localized in the rachillas and the pith area. Genotypes with the highest field resistance to *A. flavus* showed the lowest amount of GUS expression in both embryos and cobs.

The susceptible inbred SC212m, the resistant inbred Mp420, and their reciprocal crosses were inoculated in 1998 with an *A. flavus* strain (GAP-2) transformed with the *Aequorea victoria* GFP vital marker to detect fungal growth in maize kernels and cobs. Black light was used to reveal GFP activity (fluorescence) in these tissues. GFP activity was observed 7 days after inoculation. A high percentage of kernels with fluorescence was found in the susceptible line and in the cross with SC212m as female parent. Fluorescence was concentrated in the endosperm tissue rather than in the embryo tissue.

SDS-PAGE was used to determine if there were differences in cob, embryo and endosperm proteins among inoculated and uninoculated control ears of resistant and susceptible genotypes. Although the protein profiles were generally similar, a protein of approximately 36 kD was less abundant in the susceptible line than in the resistant line. This protein was detected in the F_1 progeny when proteins were extracted from 56 day-old embryos inoculated 14 days after pollination. Its synthesis did not increase beyond the constitutive level after inoculation with *A. flavus*. The abundance of this protein may differ depending on genotype and developmental stage of the embryo.

A 62 kD band in uninoculated cob tissues was detected only in the resistant line Mp420, or in crosses with Mp420 as female parent. This cross exhibited little or no GFP activity in the embryo, whereas the reciprocal cross did. A preformed protein about 33 kD was found in both inbreds and hybrids. This protein increased in the susceptible hybrid after inoculation. After inoculation with *A. flavus*, two proteins of approximately 25 and 28 kD were more abundant in the cobs of the susceptible genotype during ear development.

USE OF AN OMTA(P)::GUS REPORTER CONSTRUCT TO EVALUATE CORN FOR RESISTANCE TO AFLATOXIN ACCUMULATION

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Aflatoxins are the most carcinogenic naturally occurring compounds known. Preharvest contamination of corn, peanuts, cottonseed, and tree nuts with aflatoxins are of major concern. Effective control strategies are not available to eliminate aflatoxin accumulation. A preferred strategy for control is resistant varieties, but no resistant varieties are commercially available. To better understand the molecular biology of aflatoxin biosynthesis, and resistance to its accumulation in plants, we are developing and utilizing both genetic and molecular tools. One powerful tool for understanding the factors that affect growth and aflatoxin production by *A. flavus* are gene reporter systems. We previously showed that a reporter construct containing the promoter of the *A. flavus* B-tubulin gene fused to the *E. coli* β-glucuronidase gene (GUS) is a reliable tool to measure fungal growth in corn kernels. Both laboratory and field studies have shown that colonization of corn kernels by *A. flavus* can be monitored easily and accurately by GUS expression. Here we report the construction and analysis of a new reporter gene construct utilizing the *omtA* gene promoter of the aflatoxin biosynthetic pathway. We show that expression of GUS activity by this construct (*omtA*(p)::GUS) parallels that of aflatoxin accumulation in culture. In transformant GAP26-1 harboring this construct, aflatoxin production and GUS expression on sucrose-containing medium show the same temporal pattern of induction. We provide further data to show that GUS expression and aflatoxin accumulation are correlated in corn kernels inoculated with GAP26-1. The aflatoxin content of corn genotype GT-MAS:gk either wounded or non-wounded was correlated with GUS activity expressed by *A. flavus* transformant GAP26-1. Thus this construct should prove to be a valuable tool to monitor the expression of an aflatoxin pathway gene and to evaluate corn genotypes for their resistance to aflatoxin accumulation.

GENETIC ANALYSES OF AFLATOXIN RESISTANCE FROM TEX6 CORN LINE

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We are identifying QTL associated with pre-harvest resistance to ear rot and aflatoxin production by Aspergillus flavus in corn. Molecular marker and phenotypic data analyses have been completed on three F3 mapping populations (B73 x LB31, B73 x 75-R001, and Mo17 x Tex6). Previous studies have identified Tex6 as the most promising source of resistance to aflatoxin production. Mapping results from the Mo17 x Tex6 population will be presented. Single factor analyses indicate several chromosomal regions significantly associated with resistance to either ear rot and/or aflatoxin production. Interval mapping with Mapmaker/QTL indicates one QTL for aflatoxin in 1994 and three QTL for aflatoxin in 1995. Individual markers generally account for 4-7 % of the genetic variation, resulting in multiple regression models explaining 27 % (1994) or 42 % (1995) of the variation. Most markers are associated with only ear rot or aflatoxin production. This information, along with correlation analyses, suggests resistance to ear rot and aflatoxin production is at least partially under separate genetic control. The objective of this study was to identify QTL associated with resistance to aflatoxin production in the Mo17 x Tex6 F3 population.

POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR AFLATOXIN SYNTHESIS IN CROPS

PLATFORM PRESENTATIONS

PANEL DISCUSSION

Panel Discussion Title: Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Synthesis in Crops

Panel Members: Robert Norton (Chair), Deepak Bhatnagar, Nancy Keller, Bruce Campbell, Charles Woloshuk and Gary Payne

Summary of Presentations: The efficiency with which available carbon sources are utilized by *Aspergillus flavus* and *A. parasiticus* for growth and aflatoxin formation is a critical aspect of the aflatoxin problem. Several of the speakers in this years session reported on regulatory and metabolic factors involved in this key process, as well as plant compounds which appear to be able to reduce the level of infection by other means.

Bhatnagar (Bhatnagar, Chang et al.) described two cloned genes (*hxt*A and *glc*A) Those products are expressed in aflatoxin producing media: *hxt*A appears to be involved in sugar transport and *glc*A in release of glucose from complex sugars. Regulation of both activities could have dramatic effects on growth and toxin amount.

Woloshuk (Woloshuk and Fakhoury) reported that degradation of starch in corn kernels by fungal enzymes appeared to be the source of the aflatoxin-inducing sugars glucose, maltose and maltotriose. This was supported by a mutant of *A. flavus* NRRL 3357 with a disrupted a-amylase gene (*amy*1) which produced aflatoxin in embryo-wounded kernels, but not in endosperm-wounded kernels. Screening for amylase specific inhibitors may be warranted since inhibiting the activity of a-amylase appears to be an effective strategy for controlling aflatoxin production.

Triglycerides are a major fungal carbon source in oil seeds and seed tissues such as corn germ. Norton reported that over twice as much aflatoxin is produced from oil as from an equivalent weight of glucose. But the fatty acids composing triglycerides were not all equally productive: cis-unsaturated 16- and 18-carbon fatty acids allowed the highest toxin production in Norton's assays. Toxin production from only two fatty acids, oleic and linoleic acids, could be inhibited by certain sugars, glucose and sucrose among others. A broad survey of sugars for this characteristic showed that, of common plant sugars, xylose was the most efficient inhibitor of aflatoxin production from corn oil. Others, such as talose and glucosamine, were very effective in preventing growth.

Keller presented evidence (Calvo et al.) that linoleic acid and certain of its seed lipoxygenase (LOX) derivatives (9S- and 13S-hydroperoxylinoleic acid) are involved in early spore maturation and increased levels in *A. flavus* and *parasiticus*, as well as sclerotium production in *A. flavus*. Involvement of these compounds in the response of various peanut tissues to infection, methyl jasmonate and wounding was also shown (Burow et al., poster).

The ability of other secondary plant products to affect growth and aflatoxigenesis was emphasized by Campbell in results demonstrating significant inhibition of germ tube growth of *A. flavus* by four naphthoquinones which occur in walnuts. Only trace levels of toxin were present in growth medium using the variety 'Tulare' which has high levels of these compounds. Volatiles produced by insect wounding of commercial walnut trees play an important role in attracting the codling moth, a major pest. Two terpenes appeared to differ between susceptible and resistant varieties. Volatiles from an orchard yeast (methyl butanol and phenyl ethanol) were antibiotic. Another volatile was found which represents the first instance of a female attractant.

Payne presented results that showed that the resistance to *A. flavus* infection shown by the corn line Tex6 is due, at least in part, to two or more chitinases present in this line. This suggests that chitinases have potential to accelerate efforts to breed for resistance in corn.

The 1998 drought in the southwestern United States and severe problems with aflatoxin contamination in corn have emphasized the strong involvement of stress in aflatoxin incidence. Bhatnagar et al. suggested that a key aflatoxin pathway regulatory gene, *aflR*, may respond to stress effects in seeds through changes in the form of nitrogen in seeds (ammonium favoring toxin production, nitrate inhibiting), seed pH changes and temperature effects acting directly on the locus or its product. Payne showed that the *aflR* product is bound at a site which is required for glucose induction of aflatoxin biosynthesis. This site is upstream from the aflatoxin biosynthesis gene cluster. Payne also described three *A. flavus* reporter gene constructs useful for measuring and detecting resistance to fungal growth and aflatoxin production, including one using jellyfish green fluorescence protein.

Summary of Panel Discussion: Neil Widstrom asked Deepak Bhatnagar if he had any plan for utilizing the results presented on factors which affect aflatoxin production through the *aflR* gene? The reply was no, since their objective was to obtain the information, and make it available to breeders and genetic engineers developing resistant varieties. Another participant asked if adding ammonium later in the growing season would have adverse effects on aflatoxin levels, since the form of nitrogen affects the production of aflatoxin in the laboratory. It was noted that this year drought-stricken corn from fields producing extremely low yields of 10-15 bushels tended to have lower aflatoxin levels than ones producing 50-70 bushels. Gary Payne and Bhatnagar suggested that the nitrogen balance in the seed is highly important, and that there is no strong evidence that it can be affected markedly by what is applied to the field; but the question could not be answered with certainty.

In response to Cheng Ji's question Gary Payne responded that the *aflR* gene product is not a universal regulatory protein, and nor is it specific for the aflatoxin/sterigmatocystin pathway. However it is a member of, and thus has commonality with, a family of regulatory proteins that share that same zinc-binding domain. Payne noted that the gene was originally cloned by Charles Woloshuk by complementing the mutant. Ken Davis asked whether a

correlation had been seen between chitinase levels and fungal growth or aflatoxin level, and Payne replied they had not had time to do that study yet.

Hal Gardner asked if the plant-produced lipoxygenase products (9S- and 13S-hydroperoxylinoleic acid (13HPOD)) were metabolized by fungi. Nancy Keller said they hadn't yet determined this, but since *Fusarium* has been reported to have the components for the 13HPOD pathway, perhaps *Aspergillus* might have the pathway also. In answer to a question if lipoxygenases (LOXs) and chitinases are regulated by host plant drought stress or temperature, Keller said that LOXs are induced by drought and stress but it was not known whether they respond to temperature changes. Woloshuk noted that there was evidence that salt stress induces many pr (plant resistance) proteins, one of which is chitinase, and therefore chitinases probably would be induced by osmotic stress. A later questioner asked Keller if she thought the greater aflatoxin production in years with more insect damage was related to the signal transduction pathway initiated by LOX responding to tissue damage. She said that this was not known but probably should be examined. In response to another question about the identify of the signal for inducing LOX, Keller stated that the end product of the 13-LOX enzyme induced LOX.

In answer to a question on their plans for the aflatoxin transporter gene which they had reported finding, Bhatnagar replied that it was only recently cloned, and they they have no definite results yet in their efforts to disrupt the gene. They hope to determine if shutting down the gene will inhibit aflatoxin production by feedback inhibition. Since the aflatoxin transporter gene is a surface protein. it can be investigated more easily. He noted that aflatoxin is not retained in the cell following synthesis, nor is it taken up by the fungus from the medium to any extent. Norton later asked Bhatnager how he accounted for the fact that several studies had shown appreciable amounts of aflatoxin in the mycelium. Bhatnagar replied that careful studies in Joan Bennett's lab as well as his own studies had shown that only about 15-16% of aflatoxin was present in the fungal cells, and that was probably freshly produced. Other studies of the aflatoxin intermediates, sterigmatocystin and O-me-sterigmatocystin. have shown release rates of 50-60%. These two compounds can also be taken up again. so there may be constant exchange. Other pathway metabolites are retained 100%. In response to the question how these studies in liquid cultures relate to in vivo conditions, Bhatnagar observed that that would be quite difficult to estimate, since even in liquid culture such studies required extensive work with radiolabeled compounds.

In response to a question about the effect of water activity on growth and aflatoxin production. a discussion between Keller. Bhatnagar and Woloshuk resulted in the following observations: 1) Aflatoxin production tends to acidify the medium and production tends to increase with decreasing pH but certain salts. such as sodium nitrate or nitrite. are able to maintain a higher pH and may. by this means. be associated with less toxin production.
2) A mutant *A. parasiticus* strain in Keller's lab which over-expresses the *pacC* gene (involved in pH regulation) becomes alkaline and produces very little aflatoxin but it also grows poorly. making it difficult to tell which factor is involved.

3) In most studies the effect of water activity on aflatoxin production has not been separated from effects on growth. High water activity favors aflatoxin production and even drought-stressed corn has a relatively high water content--therefore low activity is unlikely to increase toxin. Higher oil content in a seed decreases the amount of water required for fungal growth.

Norton was asked what happened when *A. flavus* was grown on 2-deoxy-glucose and what level of corn oil was used in the medium. He replied that the fungus didn't grow at all, either when 2-deoxyglucose at 2% of medium was the sole carbon source or when it was added to medium containing 10% corn oil.

Regarding whether any of the proposals or mechanisms presented in this session would, if implemented, have decreased the aflatoxin problem in the Southwest this year, Woloshuk thought some of the work presented could have decreased insect damage, and perhaps some of the antifungal compounds might have had a positive effect. Woloshuk asked what factors people from the affected area thought were responsible for this years outbreak; e.g., was insect damage the principal factor? temperature? a combination of both? or was something else involved? A member of the audience commented that the growing season had a very good start in spring and looked like a bumper crop until the flowering stage when a stretch of high temperature days lasted into the grain-fill stage. He noted that there were probably well over 100 consecutive days with a temperature of over 95 degrees and that this was also the period without rain. It was noted that the smoke resulting cloud cover, from large forest fires in Mexico had introduced another variable this year which, although it tended to partially offset the lack of rain, had exacerbated the heat problem. Widstrom noted that he had found a correlation between insect damage and aflatoxin level primarily in years when the damage was great and this was related to two factors: 1) insect activity increases with increasing heat and, 2). the lack of available water makes the water-rich corn more attractive to insects--leading to greater damage than might occur in the absence of drought. He noted that army worms will go directly through the husk in dry years to obtain the moisture in the ear. A similar pattern has been seen with peanuts where soil temperature is a critical factor. Henry Keller, a grower from Castroville, Tex., mentioned that he had not had a big problem with corn earworm this year nor was there the usual correlation between insect damage and aflatoxin in his area. He noted that aflatoxin levels of dry land corn were low, but the levels in his irrigated corn were much higher. However his area did not experience the extended high temperatures that characterized other parts of the state. Doug Park observed that for the first time Louisiana had a major problem with aflatoxin in corn and cottonseed; however the contamination in cotton was probably not due to the boll weevil since this insect is mostly controlled in Louisiana. Another participant commented that during May to July, Louisiana experienced the lowest rainfall and the highest temperatures in 110 years and that night temperatures were high at same time.--the lows were in the low 80's--which, it was emphasized, would stress irrigated corn.

MOLECULAR BASIS FOR ENVIRONMENTAL EFFECTS ON AFLATOXIN PRODUCTION

Deepak Bhatnagar, Perng-Kuang Chang, Jiujiang Yu, Kenneth C. Ehrlich and Thomas E. Cleveland. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

Southern corn growers, already staggered by the withering heat and drought, were dealt another blow this year when aflatoxin was discovered in a large percentage of the corn that matured. In light of this event, it is important to examine the direct or indirect effects of environment on the toxin biosynthesis. An attempt has therefore been made here to relate, in molecular terms, the effects of sources of physiological stress on the onset of aflatoxin synthesis. The most significant environmental factors that influence toxin synthesis are the availability of adequate carbon and nitrogen sources, pH, temperature, water activity, and plant metabolites.

Carbon Source. Highest amounts of aflatoxin are produced when the fungus invades the seed embryo. Highest percentage of simple sugars (glucose and sucrose) are present in the embryo relative to other parts of the seed that contain higher amounts of complex carbohydrates. Aflatoxin biosynthesis has been known to be induced by simple carbohydrates, such as glucose, sucrose, fructose and maltose, but not by complex carbohydrates such as lactose or starch. Glucose sources that cannot be easily hydrolyzed fail to induce aflatoxin. In spite of the fact that the carbon source may be one of the most significant factors in aflatoxin production, relatively little is known about how carbohydrates play a role in this process. Recently, two genes (*hxt*A and *glc*A) have been cloned in our lab (see figure) which could provide insights into the mechanism of carbohydrate regulation of toxin synthesis. These genes are located on the gene cluster and have been determined to be expressed in aflatoxin conducive media. The *hxt*A gene product would have the properties to transport simple sugars such as glucose across membranes. The *glc*A gene product would release glucose from side chains of complex sugars (such as amylopectin in seeds).

Nitrogen Source. Nitrogen supply is usually not a limiting factor for U.S. crop development. But pools of amino acids, dependent on nitrogen, are reported to be important in regulation of toxin formation. Nitrogen source has been demonstrated to play an important role in toxin synthesis. Under drought stress conditions it is not clear if the nitrogen in seeds is present in the nitrate or the ammonium form. Nitrate repression of aflatoxin synthesis has been well documented, whereas nitrogen supplied as ammonium in media supports toxin formation. We have earlier demonstrated that *afl*R, the regulatory gene is rendered non-functional in the presence of nitrate; but the presence of multiple copies of *afl*R in the fungal cell can overcome the nitrate suppression. We have demonstrated that the nitrate effect may not be mediated by pH shifts, but by regulatory elements induced in the nitrogen metabolism gene cluster of the fungus. The different effect of ammonium and nitrate on this gene cluster could alter the binding of aflR, the aflatoxin pathway, regulatory protein, to specific regions of the aflatoxin biosynthesis pathway gene cluster. This could result in production or suppression of toxin synthesis, depending on the type of nitrogen source.

pH Effects. Fungi are capable of growing over a wide pH range. Under drought stress, physiological shifts in plants could certainly result in some pH changes. It has been established that aflatoxin synthesis optimally occurs in the pH range of 3.5-5.5. Regulation of fungal metabolism by pH is not well understood, but a gene *pacC* has been recognized as a genetic factor that responds to pH shifts through a signal transduction pathway that senses external pH. Penicillin synthesis has been found to be regulated by this mechanism. We have determined that the *afl*R promoter region has a site for binding of the PacC protein. A mutation in this binding site or deletion of the site enhances aflatoxin production suggesting that interaction of the PacC protein with this site may have a negative effect on the regulation of the toxin biosynthetic pathway.

Temperature. Neither high temperature nor drought stress alone will lead to increased concentrations of aflatoxin. High maximum and high minimum daily temperatures especially during periods of high net evaporation are more important for the development of aflatoxin than humidity or average precipitation during the same period (as was the case for southern corn this year). The ideal temperature for aflatoxin production is 29-30°C. Aflatoxin production is significantly reduced at temperature below 25°C, but completely inhibited above 37°C. It was found that the expression of the pathway regulatory gene *afl*R and other pathway genes paralleled the pattern of effect of various temperatures on aflatoxin synthesis. This suggests that the temperature modulation of toxin synthesis is the result of the effect of temperature on the genetic regulation of the pathway, rather than a physiological effect.

Water activity. Drought stress can increase the percentage of seed infected. The higher the water activity the better it is for fungal growth and toxin synthesis. But at water activity below 0.85, the growth of the fungus and its spore germination rates are considerably slowed. At water activity between 0.70-0.75, growth and spore germination completely cease. Higher aflatoxin production under drought conditions (lower water activity) may be due to a physiological shift from primary metabolism (growth) to secondary metabolism (toxin synthesis) and an activation of the aflatoxin pathway gene cluster.

Plant metabolites. It has been reported (Nancy Keller) that plant metabolites such as 13-hydroperoxy linoleic acid (13-HPODE), a product of soybean lipoxygenase, reduced mycelial weight, decreased the level of expression of the aflatoxin pathway genes, and decreased mycotoxin biosynthesis *in vitro*. In our lab, we have demonstrated that cotton leaf volatiles such as hexanal completely inhibit fungal growth and consequently aflatoxin synthesis. Other volatiles such as 1-methyl-butanol and 1-pentenol either inhibit or enhance toxin synthesis, respectively, without affecting fungal growth. The effects of these latter volatiles appear to be mediated through their effects on the expression of pathway genes.

INHIBITION OF AFLATOXIN B_1 PRODUCTION IN *ASPERGILLUS FLAVUS* NRRL 3357 BY SUGARS, CAROTENOIDS AND RELATED COMPOUNDS

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In corn the germ is the major site within the kernel for toxin synthesis and contains 35-45% triglycerides (TGs). Other host plants for *Aspergillus flavus* also have high levels of TGs in their seeds. On a weight basis *A. flavus* grown on TGs composed of oleic (tri 18:1) and linoleic (tri 18:2) fatty acids produces over twice as much aflatoxin B_1 (AFB_1) as on glucose or sucrose. Last year we showed that glucose and sucrose could inhibit AFB_1 production from TGs by up to 65%. Therefore the effect of other seed oil fatty acids, as TGs, and the interaction with sugar is of interest since fatty acid composition could affect the amount of AFB_1 produced in infected kernels. The effect of TGs composed of single fatty acids ranging from 6 carbons to 18 on growth and AFB_1 production by *A. flavus* NRRL 3357 was evaluated. Even-numbered Saturated TGs from tri 6:0 to tri 18:0 and the unsaturated TGs 14:1, 16:1, 18:1trans, 18:1cis, 18:2 and 18:3 were tested at 5% of medium with or without 5% glucose added. All experiments used the suspended disc assay method previously described (Mycopathologia 1995, 129:103).

Results showed that most unsaturated TGs were poor substrates for growth and toxin production when used as the sole carbon source. Growth and toxin production were highest for tri 8:0 which produced 50 ug/ml AFB_1 and 10 mg/ml mycelium as compared to ca. 140 ug/ml and 14 mg/ml respectively for corn oil, however 6:0 and 12:0 to 18:0 TGs produced negligible amounts of toxin and smaller amounts of growth. The addition of glucose stimulated AFB_1 production and growth additively. Results for tri 18:1trans were similar to saturated TGs. The unsaturated TGs were different: 14:1 showed no change in toxin and a small increase in growth with glucose and tri linolenate (18:3) was similar. Tri 16:1, 18:1cis and 18:2 all showed high levels of toxin production without glucose, a 30-40% decrease in AFB_1 with the addition of glucose and small decreases in growth. Nine seed oils were examined for growth and AFB_1 production on 10% oil and 10% oil plus 2% glucose. Results ranged from a high of nearly 300 ug/ml for rice bran oil to 175 ug/ml for coconut oil. The addition of glucose produced nearly the same inhibition of AFB_1 (and a small decrease in growth) for all oils except two: coconut and canola. Coconut oil AFB_1 was inhibited less and reflected its high level of saturated fatty acids; growth was slightly higher as expected. Canola was inhibited more than expected and this appears to be partly due to its high level of oleic acid, which is inhibited more by glucose than linoleic acid.

Inhibition of AFB_1 by glucose suggested that a survey of additional sugars might yield compounds with even more activity. Accordingly ca. 45 sugars and related metabolites were tested with 2% test compound as the carbon source or 2% compound plus 10% corn oil (CO). The first group yielded no "super" sugars which markedly exceeded glucose in

growth or AFB_1 synthesis. Most supported at least limited growth but not toxin synthesis. However there was a wide range of activities for the 2% sugar/10% CO group. Of those which had little effect on growth, D-xylose was most active (all sugars are D unless noted), producing 8% of the control (10% CO without sugar) for AFB_1. Other sugars in this group with significant activity were altrose (24%), glucose (34%), mannose (30%) and sucrose (38%). The other compounds of interest were those sugars which did not support growth by themselves and visually completely inhibited growth and toxin production on oil. These compounds were talose, glucosamine, 2-deoxy- and 6-deoxy-D-glucose.

Corn carotenoids and related compounds were shown in 1997 to be effective AFB_1 synthesis inhibitors and additional related compounds were examined to see if structural modifications could yield information on the molecular features involved in this type of inhibition. Beta-ionone (b-Ion), and carotenoids incorporating this moiety (beta-carotene, zeaxanthin), are ca. 6X less inhibitory than alpha-ionone (a-Ion) and a-Ion type carotenoids (alpha-carotene, lutein). Pseudoionone, non-cyclic b-Ion, showed low inhibition at lower concentrations but became more active at higher levels and at 347 ug/ml was ca. 3X more inhibitory than a-Ion. Methylation of a-, and b-ion at the 1 or 3 positions preserved the order of activity of the compounds (a-I > b-Ion) and seemed to increase their overall activity somewhat. The possibility that di-, and triketide compounds might be able to affect aflatoxin synthesis was investigated with several compounds of this type: 2,4,6-heptatrione was the most active; it inhibited AFB_1 by 86% at 265 ug/ml but had little effect on growth.

In summary it's clear that aflatoxin production from seed oils can be affected by oil source and may reflect the underlying fatty acid composition in some cases. The presence of specific sugars can sharply decrease toxin production from oil. Whether it's possible to use this information to select for seed lines with altered aflatoxin production potential is unclear since most oils are sold for the specific characteristics that their composition gives them. The increasing emphasis, for health reasons, on monounsaturated oils, however, may result in somewhat greater inhibition from sucrose in the germ in high oleic acid lines. Xylose is a normal component of the cell walls of corn and other plants. If corn lines could be found which accumulate xylose, especially in the germ, they should have markedly lower toxin levels. Manipulating the composition of primary metabolites in seeds to allow growth of the fungus while inhibiting toxin formation should put less selection pressure on the fungus and, as a toxin reduction strategy, could last longer than selection for decreased fungal growth. There is definitely potential for unobjectionable compounds of this type in lowering aflatoxin production by *A. flavus*, especially in oil-rich environments.

ROLE OF POLYUNSATURATED FATTY ACIDS ON *ASPERGILLUS* DEVELOPMENTAL PROCESSES

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Aspergillus spp. are frequent seed-colonizing fungi that produce carcinogenic mycotoxins, aflatoxin and sterigmatocystin. The species of this genus have the ability to develop conidiophores (i.e. asexual spores) and overwintering bodies (i.e. cleistothecia and sclerotia). The extracellular signals directing asexual or sexual development are not known for Aspergillus spp. with the exception of a sporogenic factor, called psi factor, identified in A. nidulans in 1987. Psi factor is a linoleic acid derivative that governs the relative development of cleistothecia or conidiophores in A. nidulans. Considering the role of psi factor in Aspergillus development and the fact that the primary carbon sources for seed-infesting Aspergillus spp. are fatty acids it is important to know if any seed fatty acids could be acting as potential sporogenic factors. We have found that development of all three of these structures in *Aspergillus nidulans*, Aspergillus flavus and Aspergillus parasiticus are affected by psi factors, linoleic acid and linoleic acid seed-derivatives, hydroperoxylinoleic acids (9S-HPODE and 13S-HPODE). Specific morphological effects of these fatty acids include inducing precocious and increased asexual spore development in *A. flavus* and *A. parasiticus* strains and altered sclerotial production in *A. flavus*. In *A. nidulans*, the asexual to sexual spore ratio was altered by all three compounds in a manner similar to that of the A. nidulans sporulating factors, psi factors. Seed fatty acids primarily consist of linoleic (18:2), palmitic (16:0) and oleic (18:1) acids). In addition to these primary fatty acids, seeds also produce lipoxygenase-derived linoleic acid derivatives, 13-hydroperoxy linoleic acid (13S-HPODE) or 9-hydroperoxy linoleic acid (9S-HPODE) upon pathogen attack. These last two compounds are especially pertinent as we have previously demonstrated in liquid shake cultures that 13S-HPODE inhibited AF/ST gene expression and 9S-HPODE prolonged AF/ST gene expression in A. parasiticus and A. nidulans. The work presented investigates the hypothesis that plant derived fatty acids could function as Aspergillus developmental signals. A better understanding of the molecular mechanisms governing Aspergillus lipid metabolism could contribute to the design of control strategies to reduce the survival and spread of seed-colonizing aspergilli.

VOLATILE AND NONVOLATILE NATURAL PRODUCTS OF HOST-PLANTS AND MICROBES AFFECTING INSECT PESTS, *ASPERGILLUS* GROWTH AND AFLATOXIGENESIS IN TREE NUTS

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The US tree nut crop (almonds, walnuts and pistachios) is valued at $1.5 billion/ yr. Over 50% of these tree nuts are exported. Major importing nations (EU) set strict tolerance thresholds (<4 ppb) for aflatoxin. Reducing aflatoxin contamination and infection by aflatoxigenic aspergilli in tree nuts are important to overall exportability of tree nuts, in addition to providing a safe product for domestic consumption. Two strategies to reduce or eliminate tree nut contamination by aflatoxins include control of major insect pests and control of either *A. flavus* or aflatoxigenesis.

Research efforts have focussed on identifying endogenous natural products which confer resistance to *A. flavus* growth or aflatoxin biosynthesis in tree nuts. Comparison of almond, pistachio and walnut kernels as growth media for *A. flavus in vitro* shows that walnuts delay growth significantly but, ultimately (after 7 days) growth is complete, at which time aflatoxin production has reached a maximum. There are significant differences in aflatoxin production between various cultivars and crosses of both almonds and walnuts. The commercial walnut variety 'Tulare' is especially significant, producing only trace levels of aflatoxin when used as growth medium *in vitro*. Four naphthoquinones occurring in walnuts have been shown to inhibit germ tube formation *in vitro*, stimulating aflatoxin production at very low concentrations but inhibiting at a higher concentration. Naphthoquinones are structurally similar to the anthoquinone portion of some of the early products in the aflatoxin biosynthetic pathway. The potential for naththoquinones to act as regulators of aflatoxigenesis is being examined.

Feeding by larvae of major insect pests creates wounds to tree nut kernels allowing infection by aflatoxigenic aspergilli. Host plant volatiles (HPVs) act as cues to insects to locate host plant tissues. Codling moth (CM), a major pest of walnluts, differentially infests varieties of commercial walnuts (*Juglans regia*). CM seldomly infests noncommercial species of walnut. Almost one hundred HPVs were identified by GCMS in commercial and noncommercil walnuts. Most of these HPVs were mono- and sesquiterpenes. Grafting affects the chemotype of walnuts and influences the mosaic of terpenes produced by commercial walnuts. Two of the total terpenes were only detected in the commercial walnuts. The role of terpenes as attractants to CM and antibiotics against *A. flavus* or as anti-aflatoxigenic signal compounds is being investigated.

Antibiotic volatiles were identified in a yeast, *Candida krusei*, collected from tree nut orchards. The compounds were two isomers of methylbutanol and phenylethanol. Six, new HPVs were found to synergize pheromone attractancy to male CM. A fruit volatile was found that is a female CM attractant. This HPV is a significant discovery because of

the potential for its utilization as an attracticide, mating disruptor or for monitoring female moth populations and moth emergence. There are no female attractants know, other than this compound. for CM.

Posters are presented detailing much of the above findings.

INHIBITING AMYLASE AS A STRATEGY FOR AFLATOXIN ELIMINATION

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Metabolites in corn kernels that are involved in the induction of aflatoxin biosynthesis can be considered susceptibility factors that are inherently part of the interaction between *Aspergillus flavus* and corn. Altering the production or the availability of these metabolites through methodologies such as breeding, chemicals, bioengineering or biocontrol would ultimately lower aflatoxin production.

We previously identified an aflatoxin-inducing activity in culture filtrates of the aflatoxigenic *A. flavus* strain NRRL 3357 grown on maize kernels. The inducing activity was determined to be glucose, maltose, and maltotriose. We hypothesized that the source of these molecules was starch degraded by an amylase produced by *A. flavus*. *A. flavus* produces a single α-amylase with a pI of 4.3. Evidence suggest that α-amylase produced by the fungus plays a role in aflatoxin production in diseased maize kernels. To test this hypothesis, the α-amylase gene *amy1* of *A. flavus* was disrupted by site-directed mutagenesis.

Disruption of *amy1* in transformant T101 was confirmed by PCR, Southern and DNA sequencing analyses. Iodine staining of starch media cultures and protein gel analysis indicated T101 lacked α-amylase activity. Growth of T101 on potato dextrose agar (PDA) and Czapecks agar media was identical to the α-amylase-producing transformant (T33). However, on starch medium, T-101 grew at 30% the rate of the T33. The growth on starch was due to glucoamylase produced by the fungus. T101 produce aflatoxin when grown on media containing glucose, but unlike T33 it did not produce aflatoxin in starch medium. T101 produced aflatoxin when inoculated to embryos-wounded corn kernels, however it did not produce aflatoxin in endosperm-wounded kernels. In contrast, T33 produced aflatoxin under both circumstances. These data suggest that α-amylase has a significant role in the infection process in kernels.

Our data suggest that inhibiting the activity of the α-amylase produced by *A. flavus* may be an effective strategy for controlling aflatoxin production. We are using partially purified α-amylase from A. flavus to screen for amylase-inhibiting proteins. Sources include A. flavus resistant corn and a large collection of protein extracts from legume species.

GENETIC STUDIES ON THE REGULATION OF AFLATOXIN ACCUMULATION

Gary A. Payne. North Carolina State University, Raliegh, NC

Research effort in my lab has focused in three areas: 1) characterization of inhibitory compounds in corn inbred Tex6; 2) characterization of aflatoxin regulation; and 3) evaluation of reporter gene constructs for use in screening for host plant resistance to aflatoxin contamination. In our studies on host plant resistance we have continued to characterize the inhibitory compound from corn inbred Tex6. Our data clearly show that the inhibitory activity in Tex6 is due, a least in part, to a chitinase(s). Through gel filtration and affinity chromatography we have identified two chitinases that are associated with inhibition of *Aspergillus flavus*. Another band with inhibitory activity also was retained by a chitin affinity column, and we are currently characterizing this compound. Our focus now is to purify these chitinases, clone and sequence the genes, and use them as markers in the marker assisted breeding program. Mapping of the genes and their use in breeding for resistance will be done in collaboration with Torbert Rocheford and Donald White at the University of Illinois. Our current findings indicate that this chitinase(s) has great potential in accelerating breeding efforts for resistance to aflatoxin contamination.

Research in our lab is also underway to further characterize the regulation of aflatoixn biosynthesis. We have identified an AflR binding region between 91 and 138 bp upstream of the translational start site of *nor-1* that is required for AlfR regulation of *nor-1* transcription. This region is required for glucose induction of *nor-1* transcription. Further characterization of this region by Southwestern analysis showed that AflR binds the site TCGgccagCGA, which is similar to that reported for the *A. nidulans aflR* and the *stcU* promoter. It is interesting that the *aflJ* promoter does not contain this site even though we previously showed that overexpression of *aflR* leads to transcriptional upregulation of *aflJ*. We also know from previous studies that the AflR self-binding site is not required for *aflJ* expression. Because of this apparent paradox, we designed experiments to test whether *aflR* is required for the induction of *aflJ* transcription on media conducive for aflatoixn formation. *Aspergillus flavus* strain 649, which lacks the aflatoxin biosynthetic cluster and thus *aflR*, was transformed with an *aflJ*(p)::GUS reporter construct alone or in combination with a construct that expresses *aflR* constitutively. Only transformants that contained both *aflR* and the *aflJ*(p)::GUS construct expressed GUS in media conducive for aflatoxin formation. These data show that *aflJ*, like all other genes in the cluster that have been examined, requires *aflR* for its transcription.

On reason for characterizing aflatoxin regulation is to develop tools to identify plant genotypes with resistance to aflatoxin accumulation. Several investigators have used gene reporter constructs developed in our lab to measure resistance to fungal growth.

Last year we reported the development of three new reporter constructs. One construct, *cmv*(p)::gfp, was made by fusing a viral promoter to the gene coding for the jellyfish green fluorescence protein (GFP). Research reported at this meeting in the abstract by Alfaro et al. show that this construct is a very powerful tool to study colonization by *A. flavus*. The other two constructs we developed, *aflR*::gfp and *omtA*(p)::GUS, measure expression of aflatoxin biosynthetic genes. Research this past year has shown that both GFP expression by transformants with aflR::gfp and GUS expression by transformants with omtA(p)::GUS are correlated with aflatoxin production by these transformants. Thus, we predict that these two constructs will be powerful tools to screen plants for resistance to aflatoxin accumulation.

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POSTER PRESENTATIONS

PURIFICATION OF A SMALL PEPTIDE WITH ANTIFUNGAL ACTIVITY AGAINST *ASPERGILLUS FLAVUS*

Anne-laure Moyne, Thomas E. Cleveland, and Sadik Tuzun. USDA, ARS, Southern Regional Research Center, New Orleans, LA

The aflatoxin-producing fungi, *Aspergillus flavus* and *A. parasiticus*, present health hazards to humans and animals through the toxic and carcinogenic properties of their secondary products. Expression of antifungal proteins that have activity against *Aspergillus* spp. in the seed of transgenic plants prior to fungal proliferation may prevent accumulation of toxins in plant tissues. The success of the transgenic expression relies on availability of genes encoding antifungal proteins that are active against *Aspergillus* spp. Our program is specifically directed towards cloning genes naturally present in bacteria that encode proteins active against toxin-producing fungi. We identified a bacteria *B. subtilis* (AU 195) with a high antifungal activity against *A. flavus in vitro* and demonstrated that the major active components were secreted into the culture filtrate. The purpose of the study was to isolate the biologically active fraction of the culture filtrate and to determine the chemical and antifungal properties of the antagonistic substance produced by this strain.

The production of the antifungal compound was detected to be maximal after 7 days of bacterial culture. The 7th day culture filtrate was sequentially precipitated with 20, 40, 60 and 80% ammonium sulfate. Using a disk filter assay, only fraction precipitated with 20% ammonium sulfate exhibited antifungal activity against *A. flavus*. To characterize the antifungal proteins, the fraction precipitated with 20% ammonium sulfate was further separated with isoelectro focusing (IEF) tube gel electrophoresis. For this purpose, 1 mg of protein sample was loaded on a tube gel containing ampholytes (pH 2-8) and after electrophoresis gels were cut in 1cm pieces. Each piece (total of 15) was placed on an agar media that was already inoculated with the fungus. The gel segments containing proteins with a pI closed to 4.5 inhibited the mycelium growth of *A. flavus*. Using an in-gel activity assay, we have found that it has a very low molecular weight around 3.5 kD. Considering all these data, we have purified the peptide using a preparative two-dimentional (2-D) electrophoresis system developed by Bio-Rad Co. The first step fractionate proteins into defined pH ranges by liquid-phase isoelectric focusing in the Rotofor Cell. In the second purification step, using preparative polyacrylamide gel electrophoresis (PAGE) in the Model 491 Prep Cell, individual proteins are separated with high resolution on the basis of their size differences. The antifungal activity of these fractions was evaluated using a filter disk assay and the purity by analytical SDS-PAGE. Antifungal fraction were further separated on a 16% native gel: one lane of the gel was silver stained and the other lane was overlaid with *A. flavus* spores mixed in PDA medium. After silver staining, a major band was apparent and correspond to an inhibition zone if the gel was overlaid with PDA and *A. flavus* spores. The band corresponding to the antifungal peptide was bloted on an Immobilon CD PVDF membrane, excised and sent for amino acid analysis and sequencing. The amino acid composition reveal a high

content in cysteine and glutamic acid. An initial attempt to sequence the peptide failed most probably due to the high content in cysteine amino acid. The antifungal peptide, we describe here, appears to be different from the antifungal peptides produced by *B. subtilis* described in the literature because of its properties: it is a cysteine rich 3.5 kD acidic peptide, stable at room temperature, very active against *A. flavus* and seems to have a broad spectrum of antifungal and antimicrobial activity.

CONTRIBUTION OF MAIZE KERNEL CONSTITUTIVE AND INDUCED PROTEINS TO RESISTANCE AGAINST *ASPERGILLUS FLAVUS* INFECTION

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Maize genotypes resistant and susceptible to *Aspergillus flavus* infection and aflatoxin production were compared for differences in both inducible and constitutive proteins. Inducible proteins were studied in two resistant and two susceptible genotypes with or without fungal infection under kernel screening assay conditions. The level of a 22 kDa protein was found to increase in resistant genotypes, but not in susceptible ones during the 7-d incubation without fungal infection. The changes of two other proteins were also found different between resistant and susceptible genotypes. Western blot analysis of changes of specific antifungal proteins also revealed quantitative and qualitative differences, especially the levels of a 28 kDa chitinase-like protein and the 14 kDa trypsin inhibitor protein in dry kernels as well as their changes during the incubation. Differences in proteins were also observed between resistant and susceptible genotypes in embryo tissue of pre-soaked or soaked and infected kernels. Several constitutive endosperm and embryo proteins were also found to be associated with resistant or susceptible traits. However, more genotypes need to be screened.

To assess the contributions of constitutive and inducible proteins to resistance in kernels, aflatoxin productions were assayed in infected dry kernels or kernels that had been pre-soaked at 0 °C for 3 days or pre-soaked and freeze-thaw treated before inoculation to kill embryo. It showed that freeze-thaw treated maize kernels supported the highest levels of aflatoxin production among the three treatments. Freeze-thaw treatment increased the aflatoxin production in GT-MAS:gk and CI2 by 5 and 12-fold, respectively; and by 9 and 2-fold in freeze-thaw treated B73 and G4666, respectively when compared to dry kernels. However, the level of aflatoxin in the freeze-thaw treated GT-MAS:gk and CI2 was still several fold lower than that in B73 and G4666. The pre-soaking of maize kernels at 0 °C did not change the level of aflatoxin production significantly in GT-MAS:gk and CI2, but dramatically reduced the level (5 to 6 fold) of aflatoxin production in susceptible genotypes. This study indicated that both constitutive and inducible proteins are required for resistance against *A. flavus* infection and aflatoxin production.

NEW PLANT COMPOUNDS INHIBITORY TO AFLATOXIN PRODUCTION

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Aflatoxin is a secondary metabolite produced by some strains of *Aspergillus flavus* and *Aspergillus parasiticus*. These have the ability to invade a wide variety of crops and when the right conditions are present will also contaminate these crops with aflatoxin. Extracts from household black pepper (*Piper nigrum*) have been found to inhibit the expression of the GUS gene under the control of an aflatoxin biosynthetic promoter (*nor-1*). A TLC bioassay was used to screen for compounds that would inhibit the expression of the GUS gene. In addition to the identification of these compounds flash chromatography was used to semi-purify one of these compounds. The semi-purified compound was tested with the TLC bioassay to confirm the initial observation in regards to its inhibition of the expression of the GUS gene under the control of an aflatoxin biosynthesis gene (*nor-1*). in a similar bioassay, the compound failed to inhibit fungal growth and the expression of GUS under the control of the constitutive promoter *benA*. Identification of the structure of the purified compound is in progress. This assay is being developed to screen other sources of inhibitory compounds including peanuts showing some resistance to aflatoxin. It is hoped that the identification of inhibitory compounds may provide new opportunities to develop aflatoxin control strategies.

FUNGAL INFECTION OF PEANUT PODS BY *ASPERGILLUS PARASITICUS* DURING THE PRE-HARVEST PERIOD

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Aspergillus parasiticus is one of the main aflatoxin producers. This fungus is a typical saprophyte and opportunistic plant pathogen. Preharvest fungal infection of peanut pods has been thought to occur through wounds and/or senescencing tissue. Using a method that closely resembles field inoculation we studied the infection process of A.parasiticus on intact peanut (Arachis hypogaea) pods during the preharvest period. Our preliminary results showed that the fungus could infect the unwounded peanut pods and penetrate the intact parenchyma cells of young shell. In the shell, sclerenchyma cells were less colonized by the fungus compared to parenchyma cells. Initial invasion of the kernel could occur in any part of epidermal cells, however, mycelia were most often observed in the epidermal cells between the cotyledons and embryos. Aflatoxin could be detected in shell and kernel, but not in the seed coat. Aflatoxin tends to be produced in thinner hypha.

REPRESSION OF AFLATOXIN BIOSYNTHETIC GENES OF *ASPERGILLUS FLAVUS* BY ACETOSYRINGONE

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Aspergillus flavus produces aflatoxin B1, a potent hepatotoxin and carcinogen. Contamination of food by aflatoxin B1 (AFB_1) has been recognized as a serious health hazard to both human beings and animals. Searching for natural compounds inhibitory to aflatoxin biosynthesis in *A. flavus* may lead to new strategy for reducing AFB_1 in food chain.

Aflatoxins are synthesized through the polyketide pathway and more than twenty- five genes are involved. A regulatory gene, *afl*R, is required for the expression of several identified genes in the pathway. The first stable intermediate is norsolorinic acid (NOR). At least 16 enzyme-catalyzed steps are required to complete the synthesis of aflatoxin B1 from NOR.

Acetosyringone, a phenolic signal molecule, was demonstrated toinhibit a very early step of AF biosynthesis by Hua *et al.*. The purpose of the present investigation is to examine the effect of this phenolic compound on the gene expression of AF biosynthetic pathway using GUS reporter construct. The details of fusing GUS to the promoters of *nor* and *ver* genes were described by Feharty and Payne. The gentically manipulated *A. flavus* strains 652-2 GAP-19 and 656-2 GAP 13-22 provide a useful tool to examine factors influencing the early step, *nor* gene and late step, *ver* gene expression of aflatoxin biosynthesis.

The *nor* and *ver* genes were coordinately induced in the conducive GMS medium and repressed in PMS medium. When these two fungal strains were grown in GMS media containing 1 mM and 2mM acetosyringone both *nor* and *ver* genes were repressed significantly. The extent of repression was quantified by measuring the activity of β-glucosidase (GUS) in the crude enzyme extract.

Because *afl*R is required for the biosynthesis of the enzymes responsible for the production of aflatoxin B1, the effect of acetosyringone on AFLR protein was evaluated using Western Blot as described by Liu and Chu. The amount of AFLR protein present in the crude extract of the two strains of *A. flavus* was not affected by the addition of 1 or 2 mM of the phenolic compound in the GMS medium as determined by Western analysis. The result suggests that acetosyringone may be used to further delineate the complex regulation of aflatoxin biosynthesis.

RESISTANCE OF ALMOND AND WALNUT VARIETIES TO AFLATOXIN PRODUCTION

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Aflatoxin contamination in tree nuts is rare; however, high levels can develop in a small percentage of nuts such that a single nut can contaminate an otherwise aflatoxin-free test lot. In order to prevent high levels of aflatoxin from developing in tree nuts we are trying to identify endogenous natural products that inhibit or reduce aflatoxin contamination. We are examining varietal diversity as a potential source of natural resistance to aflatoxin production. The pistachio cultivar Kerman accounts for the majority of commercial pistachios. However, there are many almond and walnut varieties used in commercial production. We tested some of these varieties, along with breeding crosses developed by Gale McGranahan and Tom Gradziel, for their natural resistance to aflatoxin production. Aflatoxin analysis of inoculated media containing ground tree nut kernels revealed a range of susceptible and resistant cultivars for both walnuts and almonds. In general, for equal weights of nut kernels, the highest levels of aflatoxin are produced on almonds, the lowest amounts on walnuts, with pistachios in between. Even though *A. flavus* appeared to develop more slowly on walnuts, the fungal growth and sporulation were identical to the almonds and pistachios after 8 days, by which time the aflatoxin production for all tree nuts had peaked. Within the almond varieties tested there is almost a ten-fold range of aflatoxin produced. Crosses developed by Tom Gradziel had the most aflatoxin inhibitory activity (cross F7.2-9; 20μg aflatoxin) and the most susceptibility to aflatoxin production (cross F7.6-11+12; 192μg). Varietal differences in walnuts also result in variability in aflatoxin production. Only trace levels of aflatoxin were produced on the walnut variety Tulare, while the walnut with the most susceptibility to aflatoxin production (Chico, 28μg aflatoxin) produced approximately the same amount as the lowest almond variety. The Tulare walnut is promising as a source of resistance to aflatoxin production; and this high-yield variety, developed by Gale McGranahan, is already in commercial production. In order to isolate the chemical factors responsible for the inhibition in aflatoxin production, we first extracted the oil from several varieties of tree nuts and tested the defatted kernel meal for aflatoxin inhibiting activity. The defatted meal of pistachios and almonds both supported aflatoxin production, while defatted walnut meal did not. In potato dextrose agar, defatted Tulare meal inhibited aflatoxin production by 79% compared with the control. Working with Abhaya Dandekar at UC Davis we will continue fractionating the defatted Tulare meal in order to try to isolate the inhibitory factor in Tulare walnuts.

EFFECTS OF NAPHTHOQUINONES FROM WALNUTS ON AFLATOXIN PRODUCTION AND GERMINATION OF *ASPERGILLUS FLAVUS*

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Aflatoxin contamination in pistachios, almonds, and walnuts is more prevalent in insect damaged kernels. *Aspergillus flavus* is a weak plant pathogen and damage to natural barriers to fungal contamination in tree nut kernels, such as cuticular layers and seed coat, enhances *A. flavus* colonization. Undamaged pistachio hulls are resistant to *A. flavus* colonization while damaged hulls are easily colonized; however, similarly damaged and inoculated walnut hulls showed no *A. flavus* colonization.

Naphthoquinones found in walnut hulls are known inhibitors of fungal growth. Four of these naphthoquinones were shown to effect the germination and aflatoxin production of *A. flavus*. These compounds completely inhibited the germination of *A. flavus* spores in potato dextrose agar at the following concentrations: 1,4-naphthoquinone at 170ppm, 5-hydroxy-1,4-naphthoquinone (juglone) at 100ppm, 2-methyl-1,4-naphthoquinone at 50ppm, and 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin) at 40ppm. Germ tube formation was delayed up to 108 hours at concentrations lower than those required to completely inhibit germination. However, all the compounds except 1,4-naphthoquinone produced some stimulation of aflatoxin production even with a delay in germination. 2-Methyl-1,4-naphthoquinone produced the most aflatoxin stimulation--350% of control at 20ppm.

Inhibition of spore germination could explain the lack of fungal growth on inoculated damaged walnut hulls. Walnut hulls contain varying amounts of these naphthoquinones depending on the species and seasonal variation. Additional naphthoquinones present in walnut hulls will be isolated to further examine the structure-activity relationship of these compounds on the germination and aflatoxin production of *A. flavus*.

VOLATILES OF WALNUT HYBRIDS AND SPECIES BY HEADSPACE GCMS ANALYSIS: A POTENTIAL AID IN SELECTING VARIETIES RESISTANT TO DISEASE AND INSECT ATTACK

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Walnuts are protected from infection by aflatoxigenic aspergilli. The walnut kernel is surrounded by a thick hull, a shell and a pellicle. Larvae of the codling moth (CM), a chief pest of commercial walnuts (*Juglans regia*), are able to feed through these protective layers. This type of feeding damage, including respective pests of other tree nuts (almonds and pistachios), is associated with infection by aflatoxigenic aspergilli. Volatiles of walnuts may play a significant role in attracting female CM to ovipositional sites in walnut orchards. Different species and varieties of walnuts produce different odors, even distinguishable by human smell. Olfactory sensitivity of CM is much greater than humans. Noncommercial *Juglans* are seldom attacked by CM. We examined the headspace volatile constituents of commercial and noncommerical species of *Juglans* of different geographic origins and two non-*Juglans* species of Juglandaceae by GCMS. Fifty volatiles, mainly mono- and sesquiterpenes, were suitable for use in a cladistic analysis. Based on the volatile chemotypes, Neotropical *Juglans* were basal and Palaearctic *Juglans* (Asian and English walnuts) were not monophyletic. English walnuts were placed in a clade containing Nearctic *Juglans* onto which commercial walnuts are grafted. This finding suggests that grafting affected expression of some volatiles. Also notable was the relatively high number of terpenoids detected in the commercial walnuts. This may reflect breeding for higher quality walnuts richer in flavor and aroma. Terpenoids, in general, play a major role in flavor and aroma chemistry. Terpenoids are generally plant defensive of signal compounds. They can act as semiochemicals to insects or antibiotics against microbes. The differences in terpenes in these *Juglans* may explain the differences in their infestations by CM and aflatoxigenic aspergilli.

VOLATILES FROM SOME YEASTS GROWN IN VITRO INHIBIT AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS* -- GCMS ANALYSIS OF HEADSPACE VOLATILES OF ISOLATE K-1

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Because the annual tree nut crop grown in the U. S. is valued at more than one billion dollars and much is exported, increasingly restrictive rules imposed by foreign governments on imported foodstuff is cause for concern among U. S. nut producers, processors and exporters. The fungal metabolite aflatoxin produced by some species of *Aspergillus* is considered a highly hepatotoxic and carcinogenic food contaminant. Although the human toxicological evidence supporting this conclusion is debatable, the perceived danger has engendered governmental regulations limiting maximum permissible levels of aflatoxin in foods and food products. The ever-increasing stringency of these regulations (1-4 parts per billion in the EU) has created a serious challenge for the producers and exporters of U.S. food and food products and a need for novel approaches to meet the new requirements.

A simple bioassay technique based on a mutant strain of *A. flavus* which accumulates the easily visible, red-orange pigment norsolorinic acid, an intermediate in the biosynthesis of aflatoxin, has been used at WRRC to identify yeast candidates with potential for preventing aflatoxin synthesis. Certain of the yeast strains examined allowed dense growth of the mutant *A. flavus* but without formation of pigment indicating that the aflatoxin biosynthetic pathway was blocked at a stage prior to norsolorinic acid. The bioassay involves growing both the mutant *A. flavus* and the yeast on the same agar plate, but not in direct physical contact suggesting that the effective yeast strains blocked norsorolinic acid synthesis by emitting volatile metabolites.

We decided to investigate this hypothesis and to do so developed a headspace trapping method for collecting the volatile substances emanating from growing yeast. The yeast samples were grown on agar in a clean, sterile closed vessel while highly purified air entered the chamber near the bottom and exited through a trap filled with Tenax absorbent. Several days later the trap was rinsed with ether to remove the volatiles, and the solution was concentrated by distillation prior to analysis by GC-MS.

We have successfully applied the technique to yeast sample K-1 isolated from a pistachio orchard and later identified as *Candida krusei*. The volatile profile of this yeast strain was very simple and revealed two isomeric methylbutanols, 2-phenylethanol, and a trace of acetoin, in addition to large amounts of ethanol. Studies are under way to test these alcohols for antiaflatoxigenic properties and to examine other promising yeast samples.

TIMING OF AFLATOXIN BIOSYNTHESIS, TRIGLYCERIDE HYDROLYSIS AND GLUCONEOGENESIS IN A MEDIUM SIMULATING COTTONSEED

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Aflatoxin contamination, caused by the fungus *Aspergillus flavus*, diminishes the value of oilseed crops. Seed storage proteins, carbohydrates, and lipids represent potential carbon and nitrogen sources for production of aflatoxin. Oilseed storage proteins, when provided as a sole nitrogen source in combination with sucrose, stimulate aflatoxin production by *A. flavus* (Mellon & Cotty, 1998, JAOCS, 75:1085-1089). Raffinose, the cottonseed storage trisaccharide, can also support aflatoxin production. Fungal growth medium containing the three cottonseed reserve materials, raffinose, triglycerides (cottonseed oil), and cottonseed storage protein, in proportions approximating those found in mature cottonseed was used to assess a number of fungal parameters in a timed study. In addition, effects of storage lipids on aflatoxin production were ascertained in whole ground cottonseed.

Crude and refined cottonseed storage lipids (primarily triglycerides) supported growth and aflatoxin B_1 production by *A. flavus*. When lipids were removed from whole ground cottonseed by petroleum ether extraction, aflatoxin production dropped by more than 800-fold. Reconstitution of the lipid-extracted whole ground seed with crude preparations of cottonseed lipids to concentrations found in mature seed restored aflatoxin production to the previous levels.

The timed study utilizing a fungal medium with the major cottonseed storage components established an number of fungal metabolic trends. *Aspergillus flavus* appeared to selectively use raffinose in the growth medium to support initial biomass and aflatoxin B_1 production. The fungus metabolized raffinose by removal of the fructose moiety, followed by a rapid utilization of the remaining melibiose. Initial carbohydrate substrate was completely exhausted by day 2. As the exhaustion of carbohydrate approached (24h), fungal hydrolysis of triglycerides to free fatty acids began. By day 2, fatty acids reached peak concentrations. Gluconeogenesis, apparently driven by fatty acid catabolism, was evident thereafter. The fungus did not preferentially utilize major released fatty acids (linoleic, oleic, palmitic, stearic acids). *Aspergillus flavus* also produced several storage metabolites, including arabitol, erythritol, mannitol, and trehalose. Mannitol production was prominent, peaking at day 3. Selective use of carbohydrates by *A. flavus* to drive initial aflatoxin production may suggest potential control strategies for aflatoxin contamination of cottonseed.

ISOLATION OF A NEW CHITINASE FROM CORN LINE TEX6

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Tex-6 corn is an inbred line which has been shown to be resistant to *Aspergillus flavus* pathogenesis. Our lab has developed an assay which is capable of quantitatively measuring fungal growth inhibition. Inhibitory activity in Tex-6 has been associated with three bands which repeatedly appear on SDS-PAGE. Two of these bands have been shown to be chitinases. The third band has been shown to be retained by a chitin affinity column but direct evidence as to the enzymatic nature of this third band has yet to be determined. Preliminary data suggests that the majority of the antifungal activity in Tex-6 corn is associated with these three proteins. It is our hope that there is a novel quality in the Tex-6 chitinase/s which makes these enzymes more robust than other plant chitinases characterized to this point.

A PEAR VOLATILE ACTS AS A NOVEL FEMALE ATTRACTANT FOR CODLING MOTH; AND ITS POTENTIAL TO REDUCE MOTH DAMAGE AND *ASPERGILLUS* INFECTIONS

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Our objective is the development of new, effective and environmentally sound control strategies against moths that attack tree nuts and foster *Aspergillus* infections. Current means of monitoring the occurrence, population levels, and flight pattern of moth pests is solely by pheromone trap-catching of adult male moths. But it is the egg-laying of female moths that determines the damage potential of the next larval feeding generation, and the *Aspergillus* invasion capability, and thus the need for appropriate timing of control measures. Our goal is to develop a new technology of using host-plant volatile (HPV) semiochemicals that monitor or disrupt the population levels, flight pattern, mating and oviposition of female moth pests of tree nuts.

This investigation is on HPVs that attract female codling moth (CM), that attacks English walnuts. Codling moths prefer to attack apples and pears over English walnuts. We are exploiting this strong fruit preference by analyzing by GC-MS the odor composition of these fruits in comparison with walnut odor. Then in a walnut orchard context we tested the attractancy to CM of HPV compounds that are "novel" or unique to pome fruits. Odor analysis identified almost 100 volatile compounds found in pome fruit odor but not in walnut odor. A group of compounds unique to Bartlett pear odor were found to be novel and potent female codling moth attractants, and are termed here, "HPV Blend #23" for proprietary patentability. HPV Blend #23 was the sole blend of 27 blends tested to elicit CM attraction and primarily female over male attraction (by 2-3X). The female flight pattern to HPV blend #23 is correlated with the male flight pattern to sex pheromone, but slightly shifted in phase. Further, HPV blend #23 was found to be less than or equal to pheromone in potency or numbers of moths attracted. The five compounds that comprised the HPV blend #23 were tested individually and as binary combinations, and a single compound was found most attractive, primarily to females. When HPV blend #23 was tested in an apple orchard only male CM were attracted, while no moths were attracted in a pear orchard context. Furthermore, the presence of pheromone had no effect on female attraction to HPV Blend #23.

Thus, we have discovered a novel and potent, pear volatile that attracts only CM, primarily females, in a walnut orchard context. This novel HPV attractant should allow for new, effective and precise semiochemical monitoring and control systems. Potential impact and uses are: 1) monitoring female populations and emergence/flight patterns, thereby providing increased effective timing of insecticides, 2) critical monitoring of pest emergence patterns in orchards using mating disruption control, where monitoring with

pheromone traps is impossible, 3) crucial "in-season" assessment of mating disruption efficacy, by attracting/collecting females and assessing whether they have mated, and 4) as a bisexual attractant in direct control strategies of mass-trapping or attracticide (attractant bait with an insecticide: *e.g.*, BT transgenic walnuts).

EFFECT OF GLUFOSINATE-AMMONIUM ON *ASPERGILLUS FLAVUS* GROWTH AND AFLATOXIN CONTAMINATION OF CORN USING A KERNEL SCREENING ASSAY

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Glufosinate-ammonium also known as Liberty (AgrEvo USA, Wilmington, DE) is a water-soluble contact herbicide intended for application as a foliar spray for the control of annual and perennial grass and broadleaf weeds in corn and soybeans. We show this herbicide to be inhibitory to growth of *A. flavus* AF13 and *A. parasiticus* SK1. Incorporation of herbicide into PDA medium at 10-fold dilutions from 2 mg a.i./ml to 0.002 mg/ml gave complete and slight inhibition, respectively. Aflatoxin B1 production by AF13 in shake culture (A&M medium) was determined over the same four Liberty concentrations used in the radial-growth-inhibition assay. Mean ppb values for AFB1 were decreased at all concentrations. Finally, the kernel screening assay was used to assess superficial fungal growth and AFB1 production on the susceptible Pioneer 3154 hybrid in response to two concentrations of Liberty (0.2 mg/ml and 0.002 mg/ml). Kernels were dipped (30 min) either prior to inoculation or at 5 days after inoculation. All treatments were terminated after a total incubation time of 7 days. The high concentration pre-dip treatment reduced apparent colonization by approximately 60%. Neither of the low concentration treatments were significantly different from the control in AFB1 contamination. The high concentration pre-dipped kernels contained significantly less AFB1 than did kernels dipped at 5 days, however, the kernels dipped for 30 minutes at 5 days were significantly lower in AFB1 contamination than the other 3 treatments. These results suggest that Liberty herbicide application to transgenic "Liberty-Link" corn containing the pat or bar gene for herbicide resistance may ameliorate preharvest aflatoxin contamination in corn.

CROP RESISTANCE - GENETIC ENGINEERING

PLATFORM PRESENTATIONS

PANEL DISCUSSION

Panel Discussion Title: Crop Resistance- Genetic Engineering
Chair: Robert Sacher, Hunt-Wesson

Panel Members: Jeffrey Cary (Panel Chair), Arthur Weissinger, Abhaya Dandekar, Albino Maggio, Peggy Ozias-Akins, Kanniah Rajasekaran, Caryl Chlan.

Summary of Presentations: A prominent feature of this years' presentations was advances that have been made in the use of plant model systems for efficacy trials of potential antifungal/anti-insecticidal proteins/peptides. Of particular significance was the use of tobacco, both as the final host and as a model system, for determining the *in vivo* efficacy of potential antifungal agents and Bt toxin against a number of fungal, bacterial, and insect pathogens. Several speakers stressed the need to identify DNA vector components (promoters, terminators, selectable markers) that are not proprietary or can be utilized through licensing agreements that will allow for unimpeded commercializaton of transgenic plants.

Advances in Development of Transgenics Expressing Antifungals. The USDA, SRRC group (Cary & Rajasekaran) reported on the efficacy of two potential antifungal proteins/peptides using a tobacco model system for both *in vitro* and *in planta* assays. Transgenic tobacco expressing the antifungal peptide, D4E1, demonstrated significant inhibition of growth of *Aspergillus flavus*, *Verticillum dahliae*, and *Fusarium monoiliforme* using an *in vitro* germinated spore assay. In addition, *in planta* assays for severity of anthracnose (*C. destructivum*) and fireblight (*P. syringae*) demonstrated reduced lesion size. A collaboration with Mycogen/Demegen has been undertaken to use their proprietary DNA sequences for expression of D4E1 and other antifungal peptides in cotton. Similar results were obtained for assays of transgenic tobacco expressing the chloroperoxidase (CPO) gene from *P. pyrrocinia*, except that inhibition of *F. moniliforme* was not as significant. Chimeric cotton callus tissue from transformation experiments with constructs carrying D4E1 and CPO was also assayed *in vitro* for inhibition of *A. flavus* and *V. dahliae*. Though preliminary, some tissues did demonstrate antifungal activity. These tissues have been induced to form somatic embryos which will produce plantlets for further analysis and evaluation of antifungal efficacy.

Caryl Chlan at USL working in conjunction with the group at SRRC reported on her use of an *Arabidopsis* model system for the seed-specific expression and assay of antifungal proteins. Successful transformation of *Arabidopsis* with a number of antifungal genes was reported, however *in vitro* efficacy assays were problematic as control tissues demonstrated anti-*A. flavus* activity. In addition, Dr. Chlan reported on the successful transformation and expression of a maize seed trypsin inhibitor protein (TIP) that demonstrated antifungal activity *in vitro*. Future studies will focus on the utility of *in planta* assays using transgenic

Arabidopsis, expression of TIP in cotton, and also the analysis of seed-specific promoters for use in the development of vectors for expression of antifungal genes.

Dr. Art Weissinger (NCSU) reported on the continuation of studies on the efficacy of the antifungal peptide, D5C, in transgenic tobacco and peanut. He identified a number of transgenic tobacco (model system) and peanut studies that demonstrated reduced lesion size following inoculation with fungal pathogens. While several lines of R0 peanut transgenics showed enhanced resistance to *Cercospora arachidicola*, the R1 progeny did not. This was believed to be due to homology-dependent silencing of identical promoters present in the plasmid for expression of D5C and the hygromycin marker. A new vector using different promoters has been constructed and transformed into peanut and the plants are currently in the greenhouse to make R1 seed for testing. Dr. Weissinger also reported on the development of an ELISA assay using an antibody that is relatively specific for D5C. The lack of antibodies specific for these small antifungal peptides has been a bottleneck in the analysis of transgene's ability to produce these peptides. This work should enable the generation of antibodies specific for a variety of small peptides, thus allowing accurate determination of their production by transgenic plants.

Dr. Albino Maggio (a post-doc in Ray Bressan's lab at Purdue) reported on the successful cloning of a number of wheat PR-4-like partial cDNAs. A full length cDNA clone of one of these partial cDNAs, wPR4-6 (believed to encode a wheatwin1-like protein shown to inhibit *A. flavus* growth) was obtained and its protein is being produced for determination of its ability to inhibit *A. flavus*. They are also testing additional, new proteins for anti-*A. flavus* activity.

Dr. Ozias-Akins group at Tifton, GA has continued with field trials of transgenic peanut harboring the *cryIAc* gene and noted clear reduction in foliar damage by LCB in the transgenic lines. However not enough material was available to determine if aflatoxin contamination was also reduced. Peanut has also been transformed with the D4E1 gene as determined by Southern and Northern blot analysis and bioassays are to be performed soon. In addition, they have identified a number of plants that have had the soybean *lox1* gene introduced and these will be assayed for antifungal/anti-aflatoxin activity. They are also perfecting a mercury resistance selectable marker for use in future vector constructs.

Abhaya Dandekar reported on his work with Dr. McGranahan on transformation of walnut with antifungal genes. They compared expression of the *cryIAc* gene under the control of the CaMV 35S or potato ubi3 promoter and found that tissues transformed with either construct gave excellent mortality for Codling moth with an *in vitro* feeding assay. These plants have been transferred to the field for future studies. Future work is focusing on the generation of transgenic walnut expressing RIPs and lytic peptides.

Summary of Panel Discussion: The panel discussion was initiated by a general question whether constitutive expression of antifungal genes is deleterious effects to the plant. Jeff

Cary replied that initially expression will be constitutive so that the best antifungal candidates can be identified and once this is done it may well be preferable to utilize inducible promoters in an effort to ease the metabolic load on the plant. Abhaya Dandekar also stated that often yield reduction will be seen if the transgene is expressed at levels representing 1-2% of total protein but this really depends on the proteins stability and often where it is expressed or transported. Art Weissinger also brought up the concerns over phytotoxicity and yield loss that may be caused by constitutively expressed lytic peptides. He felt that it needed to be determined if secretion of these peptides was more desirable. Also expression of the antifungal genes should be engineered to keep the fungus as far away from the cottonseed as possible. He noted that the vsp promoter characterized in peanut by Peggy Ozias-Akins should provide this type of protective "shield."

Pat Dowd asked the panel if anyone had heard the report of promoter silencing due to fungal infection of transgenic plants. No one knew of this, but Art Weissinger stated that it would probably be difficult to differentiate reduced expression caused by tissue damage by the fungus, opposed to direct action on the promoter by the fungus, or some product of fungal origin.

Numerous questions were directed to the panel pertaining to regulatory concerns (especially in the European Community) with respect to approval of transgenics expressing antifungals. Jeff Cary noted that the lytic peptides D5C and D4E1 have been shown to be non-hemolytic and are broken down upon ingestion thus precluding any toxicity problem. Dr. Rajasekaran stated that haloperoxidase (HPO) production in the plant is transient and should really only result following fungal invasion which leads to the production of hydrogen peroxide, a necessary cofactor for chlorinating activity. Dr. Dandekar also stated that he would not expect toxicity of HPO generated products to be of concern as studies have shown no ill effects of ingestion of chlorinated antibiotics in animals.

In answer to the question as to why lytic peptides used in the assay of different fungi had differing levels of efficacy. Dr. Dandekar stated that the exact mechanism of interaction between the lytic peptide and fungal cell wall is not known. It is believed that the peptides interact with membrane sterols and that differences in sterol composition among different fungal species may account for the differing levels of efficacy.

Don Wicklow asked Peggy Ozias-Akins if it were possible to develop a laboratory, petri-dish type assay to determine the effects of heat and drought on expression of antifungal genes in transgenic peanut. Peggy replied that although this is a valid concern and worth looking into, this was not desirable in peanut. Not all clones will develop into plants and that some may even be chimeric in nature, thus not truly indication the effects of stress on gene expression. Peggy believed it was preferable to get the transformed materials into the greenhouse as soon as possible and perform the assays on more mature plant tissues.

USING ANTIFUNGAL PROTEINS AGAINST *ASPERGILLUS*. THE OLD AND THE NEW OF IT

Gyung-Hye Huh, Paola Veronese, Meena Narasimhan, Gipsy Paino D'Urzo, Albino Maggio, P.Michael Hasegawa, and Raymond Bressan. Purdue University, West Lafayette, IN.

Wheatwin 1 and 2 are antifungal proteins that have been isolated from wheat (*Triticum aestivum*) kernels. From their complete amino acid sequences, it has been determined that these two proteins are highly homologous to one another (97% identity) and are members of a family of plant defensive, pathogenesis-related proteins (PR-4). Both wheatwin 1 and 2 have strong antifungal activities against a wide spectrum of plant fungal pathogens as well as *Aspergillus flavus*. Partial cDNA clones encoding wheatwin 1 and 2 were isolated by reverse transcription PCR from total RNA isolated from wheat seedlings (7 to 13 day-old) without or after ethephon treatment. Oligonucleotides were designed based on the amino acid sequence and a knowledge of highly conserved regions in PR-4 family proteins. Using these primers, PCR products of 360 bp were obtained from RNA of both untreated or ethephon treated seedlings. They were found to encode three different PR-4 proteins, wPR4-1, wPR4-6, and wPR4-8. wPR4-6 (wheatwin1-like) and wPR4-8 (wheatwin2-like) differ by one amino acid from wheatwin1 and 2, respectively. WPR4-1 differed by two amino acids from wheatwin2. To obtain a full-length PR-4 cDNA clone, wPR4-6 was used to screen a λgt10 cDNA library obtained from13 day-old wheat seedlings (Clonetech). One, among three positive clones, encoded a wheatwin1-like protein. The cDNA insert of 0.8 Kb had start and stop codons, indicating that the cDNA may be full-length.

Recombinant PR-4 protein is being produced and will be tested for antifungal activity against *A. flavus*. First, cDNA encoding full-length mature PR-4 protein was cloned into pET28a (Novagen) under the control of T7 promoter. PR-4 was expressed abundantly in *E.coli* but as an insoluble protein. However, it still serves as a good antigen for antibody preparation. To obtain active PR-4 protein, two approaches are being taken. First the PR-4 will be produced as fusion protein with maltose binding protein. This system is known to improve the yield of active recombinant protein by targeting the fusion protein to the periplasm. A second approach involves the expression PR-4 from a tobacco mosaic virus-based vector in tobacco plants. This system has the advantages of allowing proper folding, to form a fully active protein.

Several new proteins with strong antifungal activity have been revealed from screens of buffered extracts from seeds of a large collection of legume species. These are now being tested against *A. flavus* and other pathogenic fungi.

We are also continuing our search for fungal cell targets of PR-5 proteins. We have isolated a gene OSS-1 encoding a serpentine receptor that appears to bind the PR-5 protein osmotin and mediate its toxic effects. As a binding target of PR-5 proteins, OSS-1 can serve as a recognition screen for site-directed and molecular evolution approaches toward improving the specific target (*A. flavus*) efficacy of PR-5 proteins.

GENETIC ENGINEERING AND BREEDING OF WALNUTS FOR CONTROL OF AFLATOXIN: PROGRESS AND POTENTIAL.

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The tree nut crops in California, including walnuts, represent a 1.5 billion dollar industry, a significant amount of which is exported and thus threatened by the new regulations regarding aflatoxin contamination. Our approach to reducing aflatoxin has two components; 1) reducing infection opportunities through insect damaged tissues by developing insect resistant plants and 2) reducing the ability of tissues to support *A.flavus* growth and toxin production. The objective of the genetic engineering approach is to identify and evaluate novel genes for resistance to codling moth, the major insect pest, and for resistance to *A.flavus*.

For engineering resistance to insect pests we have mainly focused on the *cry*IAc gene from *Bacillus thuringiensis*. We have previously demonstrated high levels of expression of a synthetically reconstructed version of *cry*IAc regulated by the CaMV35S promoter. This year we have continued work on the comparison of the expression of *cry*IAc using two promoters CaMV35S and Ubi3 (from potato). This was accomplished by screening transgenic walnut embryo lines transformed with the binary vectors pDU92.710 and pDU96.3113 expressing *cry*IAc with the CaMV35S and Ubi3 promoters respectively. Direct feeding of embryos with first instar CM larvae revealed excellent mortality with both promoters. Based upon corrected mortality data there was no significant difference between the two promoters indicating that the Ubi3 was as good as the CaMV35S promoter. Efforts this year focused on getting transformed plants into the field in two locations, one in Davis and the other at the USDA field facilities near Fresno. At this point we have 97 plants in the field in Davis and 63 in Fresno. Additional plants will be planted this fall. The plants are doing well and are 1-3 feet tall. One tree flowered this year but nuts were not produced.

Engineering resistance to *A.flavus* is still an important objective. We have already tested chitin binding proteins, SAR8.2 and PGIP. Our focus is now on RIPs and lytic peptides. The vectors for expression of RIPs have been completed and transformation of walnut has been initiated. Testing of lytic peptides from Demegen Inc/Mycogen is awaiting the formalization of research agreements to obtain gene and vector components. A major component of breeding of walnuts for resistance to insects and aflatoxin is evaluating walnut shell seal, integrity and thickness. A detailed analysis was completed of 600 seedlings that resulted from controlled pollination with about 10 nuts being analyzed per seedling. This involved data obtained over 2-6 years. Shell integrity was uniform with the exception of lines that had a Chinese selection as the parent. Strength, seal and thickness were found to increase with tree age with seal being significantly affected by year as well. Heritabilities were low to moderate (0.14, 0.15, 0.26). Seal and strength were highly correlated. Selection for good shells has been incorporated into the breeding program.

ANALYSIS OF TRANSGENIC COTTON AND TOBACCO EXPRESSING ANTIFUNGAL PROTEINS AND PEPTIDES: HALOPEROXIDASES

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Genetic transformation of cotton and tobacco were carried out with a novel gene, chloroperoxidase (CPO), from *Pseudomonas pyrrocinia.* Chloroperoxidases and other haloperoxidases convert hydrogen peroxide to much more potent antimicrobial compounds, hypochlorous acid and peracetic acid. CPO from *P. pyrrocinia* require neither a heme prosthetic group nor metal ion cofactors, thus making it an ideal candidate for use in genetic engineering of plants for enhancing fungal or microbial resistance.

We previously reported on the potential of chloroperoxidase as an antifungal agent using transgenic tobacco model system. A large number of transgenic tobacco plants producing chloroperoxidase were obtained by *Agrobacterium*-mediated transformation. Successful transformation was shown by *npt* II ELISA, PCR, Southern, northern and western blot analyses. Using the substrate, monochlorodimedon, the halogenating activity of the chloroperoxidase enzyme was also demonstrated in transgenic tobacco leaf extracts. Plant extracts from tobacco plants transformed with the chloroperoxidase gene significantly reduced the number of fungal colonies arising from germinating conidia of both *Aspergillus flavus* and *Verticillium dahliae* compared to the extracts from the non-transformed control. For example, leaf extracts from transformed tobacco inhibited the growth of germinating conidia of *A. flavus* by more than 90%. Leaf extracts from transgenic tobacco also reduced the number of colonies arising from germinating conidia of *Fusarium moniliforme*, although the results were not as significant. The transformed tobacco plants showed greater levels of disease resistance *in planta*, against a fungal pathogen, *Colletotrichum destructivum*, which causes anthracnose and a bacterial phytopathogen, *Pseudomonas syringae* pv. *tabaci*, which causes the disease, fireblight.

Parallel transformation experiments with cotton have also been carried out. Analyses to date indicate successful transformation as shown by *npt* II ELISA, PCR-Southern and by the demonstration of enzymatic activity in transgenic cotton cells. We have just begun assaying the chimeric cotton callus cells for antifungal activity. Preliminary results with extracts from selected cotton callus colonies indicate significant antifungal activity against germinated spores of *Aspergillus flavus* and *Verticillium dahliae*. Several of the transgenic callus colonies have been induced to form somatic embryos and we are in the process of regenerating plantlets for further evaluation in the greenhouse.

ANALYSIS OF TRANSGENIC COTTON AND TOBACCO EXPRESSING ANTIFUNGAL PROTEINS AND PEPTIDES: LYTIC PEPTIDES

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Our goal is to transform commercial varieties of cotton with gene constructs encoding antifungal peptides/ proteins to reduce aflatoxin contamination and the loss of lint yield and quality due to invasion by the fungus, *Aspergillus flavus*. We previously reported on the evaluation of a synthetic peptide for antifungal activity *in vitro*. The synthetic peptide, D4E1, at 1.5 μM and 12.5 μM has been shown to completely inhibit the growth of germinating conidia of *A. flavus* and *Fusarium moniliforme*, respectively, and is fairly resistant to degradation by both plant and fungal proteases. A vector construct utilizing a synthetic gene encoding D4E1 under control of the CaMV 35S promoter was introduced into cotton cells by *Agrobacterium*-mediated transformation. We have also transformed these constructs into tobacco cells as a means of quickly determining if the peptide is being expressed at efficacious levels. Previous bioassay of tobacco callus tissues transformed with the CaMV 35S-D4E1 construct demonstrated a 50-80% inhibition in the germination of *F. moniliforme* spores while transgenic cotton extracts demonstrated little if any inhibition. The level of inhibition demonstrated against *Fusarium* in the tobacco assays yielded an estimated concentration of 0.5-1.2 μM D4E1 which is approximately 10 fold less than required for inhibition of *A. flavus* spore germination (as determined by previous *in vitro* assays). These results indicated that the level of expression of the D4E1 gene under control of the CaMV 35S promoter was not great enough in either tobacco or cotton to effect inhibition of *A. flavus* spore germination (a conc. of 12.5 μM). Results of promoter strength experiments indicated that future constructs should utilize the CaMVd35S-TMV enhancer promoter in an attempt to increase expression of D4E1 to efficacious levels.

We have re-transformed both tabacco and cotton with the CaMV 35S-D4E1 construct and in addition have transformed both plants with a CaMV d35S-TMV enhancer-D4E1 construct capable of increased levels of expression of D4E1. Also transformed was a CaMV d35S-TMV enhancer -CSSP-D4E1 construct containing the cottonseed storage protein gene (CSSP) signal sequence. Inclusion of this DNA region in the construct should allow the D4E1 peptide to be secreted from the cell into the extracellular spaces. Tobacco leaf tissue obtained from *nptII* positive plantlets tested positive by PCR analysis for the presence of the promoter-D4E1 DNA. This was also true for a number of the cotton callus tissue DNA samples. Due to the small size of the D4E1 gene and lack of available specific antibody for D4E1, expression of D4E1 could not be measured at either the level of transcription or translation. However, *in vitro* and *in planta* antifungal and antibacterial bioassays were performed.

In vitro germinated spore assays performed from extracts of tobacco leaf tissue transformed with D4E1 demonstrated a high degree of efficacy against *Aspergillus flavus*, *Verticillium dahliae*, and *Fusarium moniliforme*. Slightly higher efficacy was noted for extracts of tobacco transformed with the CSSP-D4E1 construct. Preliminary analysis with transgenic cotton callus demonstrated lower levels of efficacy than tobacco against *A. flavus* and *V. dahliae*. *In planta* assays were performed on leaves of tobacco that had been transformed with either of the D4E1 constructs. Assay of *Colletotrichum destructivum* (anthracnose) lesion size showed inhibition of fungal destruction by both types of transgenes. Assay of *Pseudomonas syringae* pv. *tabaci* (fireblight) demonstrated reduced infection at lower inoculum levels for both transgenes tested.

Results indicate that a higher level of efficacy may have been achieved with the improved CaMV d35S-TMV enhancer promoter controlling the expression of D4E1. This was noted in both tobacco and to a lesser extent in cotton callus. However, it should be noted that the cotton callus lines we assayed are chimeric and therefore optimal levels of D4E1 may not be present in these tissues. A better test of the efficacy of D4E1 in cotton will be determined once cotton plants are generated from somatic embryos we now have.

GENETIC ENGINEERING OF PEANUT FOR REDUCTION OF AFLATOXIN CONTAMINATION

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Our objectives for the 1997-98 research year were to: 1) carry out a second field test of Bt (R_4) progeny for resistance to lesser cornstalk borer; 2) engineer factors produced by the host plant that retard fungal invasion or growth; 3) engineer factors produced by the host plant that inhibit aflatoxin biosynthesis; 4) enhance the commercialization potential of genetically engineered peanut.

Lesser cornstalk borer is known to inoculate peanut pods with the spores of fungi as the insect pest feeds on immature pods developing underground. By reducing the amount of scarification and penetration of peanut pods by the lesser cornstalk borer, we have hypothesized that we can reduce the pathways for entry of the aflatoxin-producing fungus, *Aspergillus flavus*, into the peanut seed. The route to insect resistance that we have chosen is the introduction of a synthetic *cryIA*(c) gene from *Bacillus thuringiensis* into the peanut cultivar, Marc-1. This is the first of our three-tiered transgene approach to reducing aflatoxin contamination. Although we have not yet produced enough material to carry out a field test of aflatoxin resistance, we have conducted our second year's field test for insect resistance on transgenic material and have observed clear phenotypic differences in foliar insect damage (by both in vitro assays and natural infestation) between lines containing the Bt toxin gene and either control, non-transgenic plants or transgenic plants transformed for hygromycin resistance but not containing an intact Bt gene. The R_4 plants which have the least insect damage in the field this year were selected by the amount of pod damage from the R_3 generation in 1997 where the percent damage on these lines ranged from 8-36%. These lines had previously been selected from the results of a foliar bioassay in the R_1 generation where larval survival was nil; therefore, it appears that early bioassay data is the most important indicator of future performance. One line which appeared to have some insect resistance based on both R_1 and R_3 bioassays does not contain an intact Bt gene even though the majority of the plants are clearly hygromycin resistant and show the presence of the hph gene by PCR. This line, however, shows considerable foliar insect damage in the field.

In an effort to reduce fungal invasion and growth, we have transformed peanut with the lytic peptide gene, D4E1 (driven by the ubiquitin promoter) from Demegen along with the tomato anionic peroxidase gene, tap1 (driven by CaMV35S). PCR and Southern analyses have shown that these plants are transformed and several of them contain only 1-2 copies of the D4E1 gene. Since the peptide gene is constructed as a translational fusion with the ubiquitin gene, we are able to detect transcripts of the transgene on a northern

blot. As expected, some lines show little to no expression, however, other lines show very high expression. We now are attempting to carry out a bioassay using leaf extracts. For reduction of aflatoxin biosynthesis introduction of the soybean lipoxygenase gene, lox1 (driven by the carrot DC3 promoter) from Nancy Keller has been accomplished and plants have been regenerated thus far from 33 out of 318 hygromycin resistant lines. Finally, in addition to exploring the use of alternative promoters for transgenes in peanut, we have initiated transformation experiments with a gene that confers mercury resistance. Selection on mercury has been successful; however, selection parameters still need to be optimized.

EXPRESSION OF THE ANTIFUNGAL PEPTIDE D5C IN PEANUT

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The primary goal of this project is to decrease the probability of aflatoxin contamination of peanuts by transforming with genes encoding antifungal peptides. Peptides with selective activity against fungi could be expressed in transformed plants to inhibit growth and reproduction of invading fungi, reducing the severity of mycotoxin contamination and the probability of its occurrence.

This is a report of progress made during 1997-1998 toward development of such a system, incorporating the proprietary Membrane Interactive Molecule (MIM¿) D5C (Demeter Biotechnologies, Inc.). D5C is one of a class of synthetic peptides modeled after the structure of cecropins. The peptide, which forms an amphipathic a-helix, is thought to insert into the cell membrane to form pores which result in osmotic shock. D5C inhibits the growth of both *Aspergillus flavus* and *Cercospora arachidicola*, in vitro. D5C showed strong activity against *A. flavus* in vitro. However, this observation left unanswered questions about the phytotoxicity of the constitutively expressed peptides, which might complicate recovery of transgenic plants. Toxic effects of the peptides could eliminate transgenic cells prior to plant regeneration, or could affect fertility of transformed plants. Further, it was not known whether observations in vitro would accurately predict efficacy against fungal growth in vivo.

A tobacco model system was used in parallel with peanut to study transformation with genes encoding D5C. Tobacco is easier and faster to transform than peanut, and transgenics can be obtained in relatively large numbers. Tobacco also has numerous fungal pathogens, facilitating test of antifungal activity. Two burley tobacco lines were transformed with D5C. Transformation was verified by Southern blots probed with the D5C, and Northern blot (mRNA) analysis. Preliminary Western blots are consistent with production of D5C. Detached leaves were inoculated with Rhizoctonia or Phytophthora.. Symptom development was delayed in transgenic leaves, and symptoms did not ever become as severe as those observed on controls. D5C was also transferred into peanut cv. NC 7. PCR and Southern-blot analysis of the primary transformants and their self-progeny demonstrated the integration of the D5C transgene.

Efficacy trials were carried out with clonally propagated R0 plants, using *Cercospora arachidicola* inoculation under greenhouse conditions as a model test system. Although symptom development was variable, several lines were identified which showed consistently enhanced resistance against *C. arachidicola* than did controls. Importantly, there was complete correlation between the presence of the D5C transgene, as indicated

by molecular analysis, and resistance against Cercospora. R1 progeny of resistant primary transformants were also subjected to both molecular analysis and efficacy testing using the Cercospora arachidicola model system. The test was arranged in the greenhouse in a randomized complete block design, with 5 replicates, where replicates were rooted cuttings derived from a single plant. Test results were scored by counting lesion number at 2 and 4 weeks after inoculation. Lesion size and % damage, a subjective measure of disease development, were also recorded at 2 weeks, and % damage was recorded at 4 weeks post-inoculation. Of these measurements, only lesion number was sufficiently objective to permit showed apparent reduction of symptoms in some lines inoculated with the test fungus. It was not possible to demonstrate a statistically significant difference between transgenic progeny and control plants. Subsequent tests of the progeny indicated that the transgene was not transcribed properly. It was determined that a likely cause for this loss of activity was homology-dependent silencing, possibly resulting from the use of two identical 35S promoters in the plasmid used to prepare transgenic peanuts. To correct this problem, a new plasmid vector was built which has an HPT selectable marker driven by a 35S promoter, while the D5C coding sequence is linked to a promoter (Ubi 7 Pro) from a ubiquitin gene derived from potato. This promoter, which shares very little homology with the 35S promoter has activity approximately equal to that of the 35S promoter, and also exhibits similar expression in various tissues. A series of transformation experiments using this new plasmid (pRC9) and peanut NC7 resulted in the recovery of 62 plants representing 16 independent events. Of these, 20 carried only the HPT gene, while and additional 11 plants carried both the HPT and D5C genes. In an effort to advance potentially useful materials toward deployment as quickly as possible, no efficacy testing was carried out. Rather, the plants have been transferred to the greenhouse to make R1 seed, and samples have been taken for measurement of D5C peptide in the plants. A major limitation has been the difficulty of detecting the D5C peptide in transgenic plants.

Antiserum raised against D5C, originally provided under an agreement with Demegen, Inc., proved to be of insufficient titer to allow detection of the peptide at levels below 40 µg in Western blot. D5C contains three thrombin cleavage sites which severely limit its antigenicity in rabbits. In order to produce a more effective antiserum for peptide detection a synthetic peptide has been designed in which cleavage sites have been eliminated. The new peptide retains all of the carboxy-terminal of the original peptide, but eliminates the cleavage sites that had limited antigenicity of the molecule. This peptide design was provided to a commercial company, Quality Controlled Biochemicals, Inc., which synthesized the peptide. This truncated version of the D5C antigen was subsequently used to raise high-titer antiserum in rabbits. Raw antiserum from these rabbits is now being used to develop more sensitive assays for expression of D5C in transgenic peanut tissue. Assay conditions were optimized to allow the detection of as little as 1.0 nanogram of the peptide by ELISA, an improvement of approximately 4 orders of magnitude over previous detection assays. This procedure made use of antiserum cross-absorbed against peanut protein extract in order to reduce non-specific

binding. Additional antiserum will be purified on Protein A columns to remove constituents that might cross-react with proteins in peanut other than the target D5C. We anticipate that purification of the antibody may enhance sensitivity and should also improve its selectivity. A preliminary test of 11 transgenic peanut lines from 3 independent events was inconclusive due to high background in non-transgenic controls. However, at least one of these samples repeatedly gave readings significantly higher than controls in replicated ELISA assays, suggesting that D5C may accumulate at detectable levels in at least some transgenics. A great deal of additional work is required to demonstrate efficacy of antifungal peptides against *A. flavus* in peanut. Results reported here offer an important new tool which can be used to monitor expression of one such peptide. This in turn offers the opportunity to test the hypothesis that the presence of such toxic peptides will retard the growth and/or prevent the replication of invading Aspergillus species, thus contributing to the elimination of aflatoxin in peanut and other crops.

GENETIC ENGINEERING OF COTTON: FOCUSING ON EXPRESSION OF ANTI-FUNGAL COMPOUNDS IN THE SEED

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Although we obtain cotton transformation and regeneration rates that are comparable or superior to those reported by other groups in industry and academia, the process is still laborious and lengthy. To efficiently identify candidate constructs with the greatest probability of conferring resistance to *A. flavus* to cotton, we are using two other plant systems to test the potential efficacy of specific gene/promoter constructs against *A. flavus*. Both tobacco and *Arabidopsis* have been used by our group to study the integration, stability, expression and efficacy of potential anti-flavus genes in transgenic plants. Both of these systems have their advantages. Tobacco transformation and regeneration is simple, efficient, and faster than cotton transformation and regeneration. Tobacco is an abundant source of vegetative material for testing expression that is either leaf specific, or constitutive. *Arabidopsis* transformation is also used to study the expression and efficacy of potential anti-fungal genes. This is our fastest transgenic plant development system - the time from transformation to seed set is about 2-3 months. If the seed is targeted as the preferred region of expression, we can obtain test results much faster than if we used cotton or tobacco systems. Because *Arabidopsis* is a small plant with simple growth requirements, it is possible to study multiple independent transformants for each construct to determine the effects of the position of integration of the new genes, the effects of copy number on expression as well as the effects of different promoters.

Over the last year, we have focused our studies on seed specific expression of anti-fungal compounds. We have expressed anti-fungal genes in the seed using seed specific promoters or constitutive promoters. We have multiple independent *Arabidopsis* transformants for 11 different promoter/structural gene constructs. Direct Southern blot analysis of putative transgenic plants confirmed the presence of newly introduced DNA in all cases. From this data, we estimate that 1-10 copies of DNA were integrated into the plant genome. Western blot analyses were performed on transgenic *Arabidopsis* to determine if the appropriate proteins were expressed. For those plants tested (chitinase, chitinase/glucanase, CSSP, and pBI 121 controls) protein was detected. Fluorometric GUS assays showed that proteins expressed in the transgenic *Arabidopsis* (in this case GUS) were functional. In the transgenic *Arabidopsis* plants, both constitutive and seed specific promoters were effective in driving the expression of the linked structural gene in transgenic *Arabidopsis* seeds. Extracts were prepared from transgenic *Arabidopsis* plants and tested *in vitro* for their efficacy against *A. flavus*. The results from these experiments were non-informative because non-transformed extracts greatly inhibited *A. flavus* growth. Similar results were obtained with *Arabidopsis* seed extracts. We plan to determine the resistance of these plants to *Arabidopsis* pathogens.

In addition to studies of potential anti-flavus gene expression in seeds, we have also studied seed specific inhibitors of *A. flavus*. The corn trypsin inhibitor protein has been shown to inhibit the growth of *A. flavus in vitro* (Chen et al. 1998). A trypsin inhibitor gene was expressed in transgenic tobacco under control of constitutive promoters. DNA was extracted from these plants, amplified with PCR and the trypsin inhibitor gene identified in the transgenic plants by Southern analyses, and the transcript was detectable in some samples. The inability to detect the transcript in all samples may have been due to degradation of the RNA, because although RNA transcripts were not detected, extracts from all these plants did exhibit anti-fungal properties *in vitro*.

We plan to finish our analysis of the efficacy of anti-fungal compounds that are expressed in *Arabidosis* tissues with *in vivo* studies. We plan to continue our efforts to transform cotton with the most promising promoter/anti-flavus gene constructs. In addition, studies of compounds that are naturally expressed in the seed such as the trypsin inhibitor will be continued in our efforts to focus on seed specific expression of anti-fungal compounds in the seed.

Chen, Z. Y., Brown, R. L., Lax, A. R., Guo, B. Z., Cleveland, T. E. and J. S. Russin. 1998. Resistance to *Aspergillus flavus* in Corn Kernals is Associated with a 14 kDa Protein. Phytopathology 88:276-281.

POSTER PRESENTATIONS

A PEANUT SEED LIPOXYGENASE GENE RESPONSIVE TO *ASPERGILLUS* INFECTIONS

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Lipoxygenases (LOX) and their products have been implicated in the response of a number of crop species to *Aspergillus* infection and aflatoxin contamination. We are interested in cloning peanut seed Lox genes as several studies have indicated that the C6-C12 products of the LOX pathway from soybean and cotton could inhibit *Aspergillus* spore germination in vitro (Doehlert et al. 1993, Zeringue et al. 1996). To examine the role of LOX and their products at the molecular level, the peanut *Aspergillus* interaction was used as representative system. A Lox gene, Pn Lox1, was isolated from an immature peanut seed cDNA library and characterized. Pn Lox1was 2.8 kB in size and highly similar to pea Lox and soybean Lox2. Based on deduced amino acid sequence, Pn Lox1 codes for a 98 kD protein and contains all the histidine moieties that are conserved in most LOXs. Results from expression studies of PnLOX1, revealed that it produced a mixture of 13S- and 9S-HPODE with linoleic acid as substrate. Northern blots showed that Pn Lox1 was expressed in immature cotyledons, roots and flowers, but not in stem and leaves of peanut. In mature cotyledons, expression of Pn Lox1 was induced by inoculation with *Aspergillus parasiticus*, methyl jasmonate and wounding. These results demonstrate that Pn Lox1 is involved in the response of peanut to *Aspergillus* infection. Production of 9 S-HPODE in peanut seeds by PnLOX1 could act to increase aflatoxin biosynthesis in A. parasiticus as it was previously shown that this fatty acid hydroperoxide extends the expression of the aflatoxin biosynthetic genes.

Previous studies on peanut seed LOXs indicated the presence of three isozymes. One was predicted to yield primarily 13S-HPODE, one 9S-HPODE and one a mixture of the two hydroperoxy fatty acids (Sanders et al. 1975). Here we report the isolation and characterization of PnLOX1 which produces a mixture of 13S- and 9S-HPODE and thus likely fits the description of the mixed function LOX described in the earlier studies (Sanders et al. 1975). We are interested in cloning peanut seed Lox genes as several reports suggest that LOXs and their products could be involved in the *Aspergillus*/seed interaction (Burow et al. 1997, Doehlert et al. 1993, Zeringue et al. 1996). Several studies have indicated that the C6-C12 products of the LOX pathway from soybean and cotton could inhibit *Aspergillus* spore germination in vitro (Doehlert et al. 1993, Zeringue et al. 1996). In vitro studies also showed that 13S-HPODE and 9S-HPODE have different effects on *Aspergillus* growth and mycotoxin biosynthesis (Burow et al. 1997). Specifically, 13S-HPODE inhibited aflatoxin (AF) gene expression and decreased AF production by *Aspergillus* spp. Conversely, 9S-HPODE extended the time of AF gene expression. These findings were significant since lipoxygenase enzymes are natural constituents of plants and are activated during biotic and abiotic stress (e.g. drought, heat, pathogen attack). This suggests that these enzymes could play an important role in reducing (13S-HPODE) or promoting (9S-HPODE) AF/ST biosynthesis in plants.

To address this hypothesis in peanut plants, we have initiated a two-prong approach to try and reduce AF contamination of peanut. First, we have obtained the soybean lipoxygenase 1 (lox1) gene - a 13S-HPODE producer - fused it to a carrot embryo promoter and have started to transform peanut with this construct in a collaboration with Dr. Peggy Ozias-Akins (Tifton, GA). Secondly, as described in this poster, we have started to clone the peanut seed Lox genes to assess both their response to *Aspergillus* infections and which hydroxy fatty acid products they produce. We find that PnLox1, encoding a mixed function LOX, is induced upon *Aspergillus* infections and thus could be an important component of the peanut seed/*Aspergillus* interaction. Since the PnLox1 produces a substantial amount of 9S-HPODE, it is possible that this hydroperoxide product could promote AF biosynthesis by *Aspergillus*. However, it also produces 13S-HPODE, a putative suppressor of AF biosynthesis so it is not clear which putative role would predominate. Also, as both compounds act as *Aspergillus* sporogenic factors (see Calvo, et al. poster), their effect on *Aspergillus* is quite complex. In order to clarify the role of peanut seed LOXs, we plan to clone the predicted remaining two Lox genes and to disrupt all three of them and then assess the null genotypes for relative resistance/susceptibility to *Aspergillus* infections and subsequent AF formation.

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MICROBIAL ECOLOGY

PLATFORM PRESENTATIONS

PANEL DISCUSSION

PANEL DISCUSSION TITLE: Microbial Ecology

PANEL MEMBERS: Maren Klich (Chair), Sui-Sheng Hua, Merritt Nelson, Joe Dorner, Peter Cotty, and Larry Antilla

SUMMARY OF PRESENTATIONS: Sylvia Hua reported that several saprophytic yeasts isolated from nut tree orchards were antagonistic to growth or pigment production of the NOR mutant of *A. flavus*. Maren Klich discussed infection of uninjured cotton bolls by *A. flavus* resulting in increased yield in infected bolls compared with uninfected controls in a greenhouse model - the first report that *A. flavus* may be metabolically active in uninjured developing cotton bolls. Peter Cotty and Joe Dorner both reported results of field experiments using non-aflatoxigenic strains of *A. flavus*. Populations of the non-aflatoxigenic strain results in competitive displacement of toxigenic strains and lower aflatoxin levels in the crops, but while the population of these applied strains decreases over the winter usually loosing its dominance in peanuts (Dorner), the strain applied to cotton field soils apparently maintains its dominance in the population through the winter (Cotty), although it may be necessary to reapply the next year. Merritt Nelson has conducted population studies of S (small sclerotia) and L (large sclerotia) strains of *A. flavus*. The incidence of the S strain does not follow field or crop boundaries indicating that other, as yet undefined, factors influence spatial distribution of this strain. He reported a dramatic, unexpected decrease in S strain occurrence in one region in late 1997 and early 1998. Larry Antilla reported on the rapid progress of development of a facility to manufacture large quantities of Dr. Cotty's biocontrol strain of *A. flavus* for commercial application to cotton fields. They plan to have enough inoculum to treat 20,000 acres by May 1, 1999.

SUMMARY OF PANEL DISCUSSION: Maren Klich was asked if she thought application of an aflatoxin biocontrol strain could potentially be used to increase yield. She reiterated that *A. flavus* not only produces aflatoxin, but is a human pathogen (usually fatal) so adding it to an environment to increase yield would not be desirable. Merritt Nelson was asked why the dramatic decrease in incidence of the S strain occurred. He had no explanation. Peter Cotty pointed out that large fluctuations in certain populations of *A. flavus* have been reported before, but the reasons for these fluctuations have not been determined. Several questions centered around a report of genetic recombination in *A. flavus* populations (D. Geiser et al. PNAS v95: 388-393) and whether or not possible recombination should be a concern in using non-aflatoxigenic strains to control aflatoxin. Among other things, the paper raised the very difficult question of whether or not different vegetative compatibility groups recombined in nature. Nancy Keller suggested cloning the genes that govern vegetative compatibility to study the problem and to use the information in practical applications. Maren Klich said several people were looking into using Geiser's method to determine if recombining populations occur in our growing areas. A number of questions were addressed to Larry Antilla and Peter Cotty regarding the commercialization of the

biocontrol strain AF36. Peter explained that the 10 lbs per acre was 10 lbs of wheat seed that had been inoculated the fungus and grown for one day then dried. Colonies this young are not sporulating. He estimated that the cost per acre for aflatoxin contamination was 20-50$, while the application cost for the biocontrol strain would be about 10$ per acre. The Arizon Cotton Research and Protection Council will be charging a fixed rate of 5$ per acre for the product and each grower will use their own application technique. It was suggested that an aerial application approach be used for area-wide implementation. This may be quite feasible since the new varieties being used have reduced the need for aerial application of other pesticides by 50%.

INCREASED YIELD IN BOLLS OF GREENHOUSE-GROWN COTTON CONTAINING *ASPERGILLUS FLAVUS*-INFECTED SEED

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Greenhouse studies are being conducted in order to begin to develop a model system to: a) study morphological and physiological events occurring during *A. flavus* infection of individual bolls, and; b) test potential aflatoxin control strategies as they are developed. To study conditions conducive to *A. flavus* infection of cottonseed in undamaged bolls, cotton plants were grown in a greenhouse in 1.5 m x 16 cm white polyvinyl chloride pipes filled with a soil mixture and subjected to various levels of water and nutrient stress. Flowers were inoculated with *A. flavus* on the involucral nectaries, the resulting individual bolls harvested, lint and seed weighed, and the seed assayed for *A. flavus* on agar plates. A boll was considered to be infected if any of the seed in the boll contained *A. flavus*. Under greenhouse conditions, seeds of undamaged bolls became infected with *A. flavus*. As in the field, only a few seed in some of the bolls were infected and virtually all of the infected seed were in bolls developing from early-season flowers. Yield in infected bolls was compared to that of uninfected bolls. Lint yield and seed weight were significantly higher in bolls containing *A. flavus*-infected seed in six of the eight experimental plantings. This unexpected result provides the first indication that *A. flavus* may be metabolically active in developing bolls and play a direct or indirect role in increasing yield.

STRATEGIES OF BIOLOGICAL CONTROL OF *ASPERGILLUS FLAVUS* TO REDUCE AFLATOXIN CONTAMINATION IN ALMONDS AND PISTACHIOS

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California is the major state in the United States for the production of almonds and pistachios with a total market value of 1.2 billion dollars. Both domestic and export markets of these nuts presently allow a maximum level of aflatoxin B1 contamination in the edible nuts to be 20 ppb. Even very low degrees of infection of the nuts by *A. flavus* can result in aflatoxin levels above the mandatory standards. Biological control to reduce the population of *A. flavus* in orchards may be useful to decrease infection and thus aflatoxin content in edible nuts.

Saprophytic yeasts, which can colonize plant surfaces for a very long period of time under dry conditions, produce extracellular polysaccharides that enhance their survivability and restrict both colonization sites for and nutrient flow to fungi. Some of the yeast species have been developed in control of post harvest fungal disease for commercial application. The potential of yeasts as effective biocontrol agents against *A. flavus* is being investigated in this project. More than one hundred strains of saprophytic yeasts have been isolated from nut-tree orchards on selective media. A visual bioassay has been elaborated to facilitate the screening of effective yeast isolates antagonistic to *A. flavus*. The NOR mutant of *A. flavus* was used in this visual bioassay because the enzyme norsolorinic acid reductase is blocked resulting in the accumulation of norsolorinic acid, a bright red-orange pigment, which is easily visualized. Because the NOR mutant produces only very low amounts of aflatoxin, it was selected for screening yeasts as biological control agents against aflatoxigenic strains of *A. flavus*. Certain yeast strains were shown to confine the spreading of NOR mutant and to reduce the red-orange pigment formation on PDA. When the NOR mutant does not produce the red-orange color, it implies that aflatoxin biosynthesis is inhibited at an earlier step in the polyketide pathway and that this inhibition would also be reflected in wild strains of aflatoxigenic *A. flavus*.

Field tests are complex and laborious and require cooperative association between the public and commercial sectors. We envision two possible ways to use these effective, antagonistic yeasts (1) applying the yeasts to almond and pistachio trees after harvesting for reduction of *A. flavus* in orchards; (2) applying yeasts to discarded hulls after processing for reduction of *A. flavus* in the vicinity of tree-nut industrial facilities.

MONITORING *ASPERGILLUS FLAVUS* COMMUNITY STRUCTURE OVER LARGE AREAS

Merritt R. Nelson,[1] Donna M. Bigelow,[1] Peter J. Cotty,[2] and Thomas V. Orum.[1] [1]University of Arizona and [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA

The prospect of managing aflatoxin contamination in Arizona cotton with the help of atoxigenic L strain isolates of *Aspergillus flavus* underscores the importance of learning more about the ecology of this fungus. In Arizona, *A. flavus* isolates can be divided into two strains, S and L, on the basis of sclerotial morphology. This classification method provides a way to study spatial and temporal characteristics of *A. flavus* community structure over large areas. The classification has particular relevance to aflatoxin management because the S strain isolates are typically (>98%) highly toxigenic. Therefore, we have framed results on community structure in terms of the percentage of isolates that are S strain (S strain incidence). Since 1994, repeated sampling of soil in Yuma County, AZ cotton-growing areas has shown that S strain incidence is patchy and that patches persist over time. Sampling of adjacent fields in July 1996, October 1996, and March 1997 demonstrated that patches of low or high S strain incidence extend beyond field boundaries and do not correspond in an obvious way with the crop in the field or with field crop sequence. These results lay the groundwork for an ongoing geostatistical approach to analysis of S strain incidence that can provide regional maps of *A. flavus* strain composition.

Data from July 1997, October 1997, and March 1998, revealed an unexpected downward shift in S strain incidence, particularly in the Texas Hill region of Yuma County. The shift is S strain incidence between July 1997 and October 1997 was evident in three ways: a temporal plot of S strain incidence in two locations sampled eleven times since April 1995; scatterplots of observed incidence vs. expected S strain incidence at 50 locations in the North Gila and Texas Hill areas of Yuma County sampled in October 1997; and regional maps of S strain incidence in the Texas Hill area based on data from April 1995 through March 1998. Although overall S strain incidence was lower, the regional spatial pattern of **relative** S strain incidence in the Texas Hill area was similar before and after the change. This supports our earlier observations that there are important spatial factors affecting strain composition, although we do not yet understand what underlies the spatial patterns.

In July 1998, 100 locations in ten cotton-growing areas in five southern Arizona counties were sampled. Analyses of these data will further the understanding of S strain incidence statewide. Our current understanding suggests a model in which shifts in S strain incidence occur at a regional scale in a way that tends to preserve patchiness and spatial structure despite background variability. With a data dependent model of expected S strain incidence, deviations from the expected can be examined for their importance to aflatoxin management. Geostatistical and GIS techniques, therefore, have been and should continue to be very useful in creating regional maps to help monitor changes in strain composition related to the use of atoxigenic strains over large areas.

EFFECT OF MULTI-YEAR APPLICATION OF NON-TOXIGENIC STRAINS OF *ASPERGILLUS FLAVUS* AND *A. PARASITICUS* ON NATIVE SOIL POPULATIONS

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Non-toxigenic, competitive strains of *Aspergillus flavus* and *A. parasiticus* were applied to test plots in different agricultural fields over a period of two to four years in experiments to biologically control preharvest aflatoxin contamination of crops. In field 1, the competitive strains were applied to 24 plots (18 × 80 ft.) that had been planted with corn, peanuts, or cotton in 1994-1997. Equivalent plots in another area of the field were not treated and served as controls. The remainder of the field was not planted, but remained in fallow. In field 2, six peanut plots (¼ acre) were treated in 1995-1997 with six equivalent plots in another portion of the field serving as nontreated controls. In field three, treatments were randomly assigned to 20 peanut plots (0.1 acre) in 1996 and consisted of five plots inoculated with 20 lb/acre of the biocontrol formulation (conidia of the competitive fungi encapsulated in a wheat-based particle), 10 plots inoculated with a 200 lb/acre rate, and five non-inoculated control plots. In 1997 the five 20 lb/acre plots from 1996 were again inoculated at the same rate, five of the 200 lb/acre plots from 1996 were inoculated again with 200 lb/acre, five of the 200 lb/acre plots from 1996 were inoculated with 20 lb/acre, and the control plots from 1996 were again non-inoculated control plots. Soil was monitored for the presence of wild-type *A. flavus* and *A. parasiticus* as well as the applied strains prior to planting each spring and prior to harvest in the fall.

In field 1, populations of the applied strains ranged from a low of 2410 CFU/g in the spring of 1997 to 41,003 CFU/g in the fall of 1995. These populations were generally higher in the fall and lower in the following spring before being reapplied. Populations of wild-type *A. flavus/parasiticus* ranged between 40 CFU/g in treated plots in the fall of 1996 to 631 CFU/g in the control plots in the spring of 1996. Populations of the wild-types were significantly ($P < 0.05$) lower in treated plots than in control plots in three of the four fall samplings, but only one of the spring samplings. Total populations of applied and wild-type strains were affected by weather conditions during the summer. In cooler, wetter years, such as 1994, the populations were much less in the fall than in the fall of hotter, dryer summers, such as 1995.

Populations in the other two fields followed the same pattern seen in field one. Populations of wild-type *A. flavus/parasiticus* were usually significantly lower in treated plots with populations of the applied strains being much higher. Peanuts in field 3 were exposed to a significant drought in the latter part of the growing season in 1997, and this was reflected in the highest soil populations seen in the entire study. In the fall sampling from field 3, 1997, average wild-type *A. flavus/parasiticus* populations ranged between 320 CFU/g in the plots treated with the 200 lb/acre rate in both 1996 and 1997 to 6662 CFU/g in the untreated plots. The average population of applied strains in those same plots totaled 42,660 CFU/g. Populations of applied strains in plots receiving the 20

lb/acre inoculum rate in 1997 were about an order of magnitude less that in the 200 lb/acre plots, averaging 1850 CFU/g.

In summary, application of the non-toxigenic strains usually (67% of the time) resulted in significantly fewer propagules of wild-type *A. flavus/parasiticus* compared with uninoculated control plots with wild-type strains averaging 4.4 times higher in control plots than in treated plots. Large increases in propagules of applied strains were found in treated plots where they outnumbered wild-type strains by an average ratio of 87:1. Levels of the applied strains averaged about 5 times higher in the fall samplings compared to samplings taken in the subsequent spring.

IMPROVING AFLATOXIN MANAGEMENT WITH ATOXIGENIC STRAINS: REQUIREMENTS AND OBSTACLES

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Procedures to produce sterile wheat colonized by *Aspergillus flavus* AF36 were improved and five thousand pounds of steam sterilized wheat seed colonized by *Aspergillus flavus* AF36 were made at the Southern Regional Research Center in 1998. This material was used in the third and final year of the experimental program being conducted under EPA Experimental Use Permit 69224-EUP-1. Analyses of soils from 25 fields sampled in 1997 were completed. Incidence of *Aspergillus flavus* AF36 was detected within these samples by vegetative compatibility analysis. Incidence of AF36 in treated fields increased from 4% in 1996 to 85% in 1997 while incidence of the highly toxigenic S strain of *A. flavus* decreased from 52% in 1996 to 4% in 1997. Significant changes in the soil communities resident in both fields adjacent to and diagonal to treated fields were also observed. In fields adjacent to treated fields AF36 incidence increased from 2% prior to treatment to 48% one year after treatment. The average quantity of *A. flavus* in soils prior to treatment did not differ from the quantity present one year after treatment. In 1998. the soils from 35 fields were sampled in order to continue assessment of long-term influences of applications and to determine incidence of applied strains in fields to be treated for the first time in 1998.

Treatments to the 1997 crop were applied between May 30th and June 18th. Displacement of aflatoxin producing strains by *Aspergillus flavus* AF36 in a manner similar to that observed in 1996 was detected and treatments were associated with reduced levels of aflatoxin in the cottonseed after ginning. Similar displacement was observed in fields treated with either a Gandy box applicator, a fertilizer spreader, or by air. However, displacement was highly dependent on the time of application ($R^2 = 0.815$) with fields treated before June 3 having significantly more displacement than those treated after June 10. The results of the 1997 tests add additional evidence that applications of atoxigenic *A. flavus* strains can be made in a manner compatible with commercial agriculture. The results also suggest that atoxigenic strains may be most useful when combined with other management tools such as Bt cotton and early harvest, and when incorporated into area-wide programs directed at reducing aflatoxin contamination throughout entire agricultural communities.

In order to better evaluate the impact of application date on atoxigenic strain efficacy, in 1998 applications were made to 12 fields between April 24 and July 9. Material was again applied by air (192 acres), with Gandy boxes (212 acres) and with a fertilizer spreader (95 acres). The material again proved to be compatible with all the application methods. In five fields, growers were allowed to cultivate after application in order to evaluate the extent to which tillage must be limited in an aflatoxin management area. Cold weather greatly delayed the crop in Arizona in 1998. This resulted in the worst crop in memory. The cool weather early in the season also prevented growth of *Aspergillus flavus* AF36 on the colonized wheat seed in those fields that received the earliest treatments.

DEVELOPMENT AND CONSTRUCTION OF A FACILITY TO MANUFACTURE COMMERCIALLY USEFUL QUANTITIES OF ATOXIGENIC STRAIN INOCULUM

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Aflatoxin contamination of cottonseed has been a severe problem for Arizona cotton producers for over 30 years. Wheat colonized by an atoxigenic strain of *Aspergillus flavus* has been applied to commercial fields in Arizona and has been shown to have excellent efficacy both in reducing the aflatoxin content of the commercial cottonseed crop and in causing long term modifications to the fungal community resident in treated areas. The long term and adjacent area influences indicate atoxigenic strain based management may provide long term reductions to the vulnerability of crops over entire regions. Such reductions would be best achieved with area-wide management programs. Due to widespread interest in the Arizona grower community, the Arizona Cotton Research and Protection Council (ACRPC) has assumed responsibility for the development of a grower owned facility for the production of commercial scale quantities of atoxigenic strain inoculum and the establishment of protocols for area-wide implementation of the technology.

Several areas of critical importance require coordinated concurrent attention in order to achieve the goal of establishing an operational atoxigenic strain production facility prior to the 1999 cotton growing season. These are as follows:

PESTICIDE REGISTRATION: With the assistance of the Interregional Research Project #4 (IR-4), the ACRPC has applied for a Section 3 registration for the use of *Aspergillus flavus* AF36 within Arizona. The submission included plans for an expanded experimental program directed at achieving EPA data requirements.

LICENSING: The ACRPC has obtained a nonexclusive royalty-free license from USDA, ARS to utilize the two *Aspergillus flavus* patents (Nos. 5,171,686 and 5,294,442) in Arizona for the production of cotton.

MANUFACTURING FACILITY DEVELOPMENT: Effective September 1, 1998, the ACRPC leased approximately 15.000 square feet of building/warehouse space to house the production equipment and associated labs and offices. On September 16th, the ACRPC took delivery of a Littleford 2000 liter horizontal polyphase blender to be utilized for sterilizing, blending, vacuum cooling, incubating and drying AF36 for large scale program use. Support equipment including a 100 hp boiler, heat exchanger, vacuum cooling system, product conveying systems along with 300 tons of wheat seed have been ordered with delivery expected on or before December 15th, 1998.

ATOXIGENIC STRAIN MATERIAL: Techniques are being developed in Dr. Cotty's laboratory to produce commercial scale quantities of atoxigenic strain inoculum.

SCHEDULE OF EVENTS:
January 15, 1999 - Completion of Phase I pilot scale production facility
February 1, 1999 - AF36 production begins
May 1, 1999 - 20,000 acre equivalents of AF36 completed for delivery to growers in defined test areas.

PROGRAM EVALUATION: A protocol for pre and post treatment soil and air sampling will be utilized as the principal means of program evaluation. Additionally, segregation of cottonseed at ginning will allow for analysis of aflatoxin levels in treatment vs. control areas.

COSTS: Total expenditures for the project in 1999 are expected to exceed one million dollars.

KEY ISSUES TO BE ADDRESSED: 1) Data requirements for Section 3 registration with EPA; 2) Optimization and automation of the manufacturing system; 3) Identification of critical elements for evaluation of the experimental use program; and 4) Development of area-wide implementation strategies.

POSTER PRESENTATIONS

ANTIFUNGAL METABOLITES, MONORDEN, MONOCILLIN IV AND CEREBROSIDES FROM *HUMICOLA FUSCOATRA* TRAAEN NRRL 22980, A MYCOPARASITE OF *ASPERGILLUS FLAVUS* SCLEROTIA

Donald T. Wicklow,[1] Biren K. Joshi,[2] William R. Gamble,[2] James B. Gloer,[2] and Patrick F. Dowd.[1] [1]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL and [2]University of Iowa, Iowa City, IA

The mycoparasite *Humicola fuscoatra* NRRL 22980 was isolated from a sclerotium of *Aspergillus flavus* that had been buried in a corn field near Tifton, Georgia. When grown on autoclaved rice, this fungus produced the antifungal metabolites monorden, monocillin IV, and a new monorden analog. Each metabolite produced a clear zone of inhibition surrounding paper assay disks on agar plates seeded with conidia of *A. flavus*. Monorden was twice as inhibitory to *A. flavus* mycelium extension (M.I.C. > 28 $\mu g/ml^{-1}$) as monocillin IV (M.I.C. > 56 $\mu g/ml^{-1}$). Cerebrosides C and D, metabolites known to potentiate the activity of cell wall-active antibiotics, were separated from the ethyl acetate extract but were not inhibitory to *A. flavus* when tested as pure compounds. This report is the first report of natural products from *H. fuscoatra*. Monorden may also play a key role in the mycoparasitic action of a taxonomically unrelated fungus, *Monocillium nordinii*, a destructive mycoparasite of pine stem rusts. These results may point to a convergence in biosynthetic pathways involving taxonomically unrelated mycoparasites.

Fungi that parasitize and kill other fungi offer a potential source of novel antifungal agents useful to agriculture and medicine. While the general concept that mycoparasites may produce antifungal agents is not new, the number of cases investigated from a chemical standpoint is surprisingly small. There is an urgent need for new sources of antifungal agents. Opportunistic fungal infections have become increasingly common in recent years and new classes of environmentally friendly agricultural fungicides are also needed. The strategy of selecting fungal parasites of *Aspergillus* as potential sources of antifungal antibiotics with activity against the same mold that causes aspergillosis in humans and poultry is unprecedented.

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SPREAD OF GENETICALLY ALTERED *ASPERGILLUS* SPECIES IN RESISTANT AND SUSCEPTIBLE CORN KERNELS

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Field studies were conducted to provide more definitive information on the process of infection and spread of *Aspergillus* species in developing ears of corn. An *Aspergillus flavus* isolate (GAP 2-4) transformed with the *Escherichia coli* GUS reporter gene and an *A. parasiticus* NOR mutant (NRRL 6111) were used to monitor fungal spread in developing corn ears. Ears of resistant (Mp313E x Mp420) and susceptible (Ab24E x SC229) corn hybrids were self pollinated and inoculated with the GUS isolate 14 or 21 days after pollination using a modified pinbar technique. Ears were harvested 7 days after inoculation and at 7-day intervals following the first harvest. After drying ears for 7 days at 38 C, 40 kernels surrounding the wound site were removed and sliced longitudinally into halves to expose the embryo-endosperm interface. Histochemical staining with X-glu (Gold Biotechnology, Inc., St. Louis, MO) was used to monitor fungal growth in kernels. Longitudinal cob sections associated with the wounded and surrounding kernels were also stained with X-glu. Additional ears of the same resistant and susceptible hybrids were self pollinated and inoculated with an *A. parasiticus* NOR mutant 7 and 21 days after pollination using a wounding (pinbar) and nonwounding (silk channel) technique, respectively. Ears were harvested 45 and 65 days after pollination to determine fungal spread by visually assaying accumulation of norsolinic acid. Infection of kernels with the *A. flavus* isolate was similar whether the ears were inoculated at 14 or 21 days after pollination. GUS activity was similar in resistant and susceptible hybrids at the first harvest. However, by 35 days after inoculation, fungal infection was 93% in the susceptible hybrid and only 62% in the resistant hybrid. GUS activity in kernels was exhibited exclusively in the embryo in the kernels. GUS activity in the embryo was concentrated in the scutellum with very little activity in the coleoptile region. GUS activity was observed in the vascular tissue in the rachis and rachilla regions of susceptible and resistant cobs harvested 7 and 14 days after inoculation. Norsolinic acid produced by the *A. parasiticus* isolate was found in very few kernels regardless of inoculation method. Most of the infected kernels were either wounded by the pinbar or by insects. The transformed *A. flavus* isolate was more useful than the *A. parasiticus* NOR mutant in determining spread of the fungus. The information gained from these studies will provide information researchers can use to develop corn with resistance to *A. flavus* infection and aflatoxin accumulation.

AFLATOXIN-PRODUCING ABILITY OF *ASPERGILLUS FLAVUS* GENOTYPES (RFLP) FROM FIELDS IN EASTERN IOWA WITH AFLATOXIN CONTAMINATED CORN AT HARVEST IN 1988

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An evaluation was made of the sclerotium- and aflatoxin-producing abilities of 259 strains of *Aspergillus flavus* isolated from soil samples collected (1988-1990) in 39 crop fields in eastern Iowa that were the source of corn that tested higher than 20 ppb aflatoxin at harvest in 1988 and 347 strains of *A. flavus* from soil samples collected (1989-1990) from corn rotation plots at three Iowa State Agricultural Experiment Stations. Sclerotia were produced by 95% of the *A. flavus* isolates from Iowa soils. Aflatoxin was produced by 49% of the *A. flavus* strains from 39 crop fields but only 20% of the *A. flavus* strains from the experimental corn rotation plots. DNA fingerprinting was performed on 88 of the aflatoxin-producing *A. flavus* strains from Iowa crop fields and 129 of the non-aflatoxigenic strains from experimental corn rotation plots. Pst1 digests of total genomic DNA from each isolate were probed using the pAF28 repetitive sequence. Fifty-six (64%) distinct genotypes (each genotype with less than 100% pAF28 band similarity) were identified among the population of aflatoxin-producing isolates. Genotype 36 was the most frequently recorded genotype from Iowa crop fields and matches the DNA fingerprint of *A. flavus* strains isolated from corn grown in a field in central Illinois as well as NRRL 19997 (= K.E. Papa h-c group 21) isolated from corn grown in Georgia. Seven of ten genotypes recorded from crop fields in two or more Iowa counties, were previously isolated from the University of Illinois River Valley Sand Field, Kilbourne, IL. Eighty-four (65%) distinct genotypes were identified among the 129 non-aflatoxigenic strains from experimental corn rotation plots. Two genotypes (GT 108; GT 303) accounted for 35% (10/29 strains) of the non-aflatoxigenic strains isolated from N.E. Experiment Station, Nashua, IA and 14% (7/52 strains) isolated from corn rotation plots at Allee Demonstration Farm, Newell, IA. The prevalence of non-aflatoxigenic strains in Iowa experiment stations may function naturally in reducing the severity of an aflatoxin outbreak.

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PRODUCTION OF A BIOCONTROL AGENT FOR CONTROL OF AFLATOXIN

Clive H. Bock and Peter J. Cotty. USDA, ARS, Southern Regional Research Center, New Orleans, LA

Aflatoxins are toxic mycotoxins produced by some members of *Aspergillus* section *flavi*. *Aspergillus flavus* may infect and contaminate many crops and crop products, including cottonseed. If aflatoxin contamination is above the maximum mandated by law the product is rejected as a food or feed and loses value. Biocontrol of aflatoxin contamination of cottonseed using atoxigenic *A. flavus* to competitively exclude aflatoxin producing strains has previously been reported, and is now in the third year of commercial scale tests. The current report describes optimized production of wheat seed colonized with atoxigenic *A. flavus*.

Adequate inoculum, moisture, temperature and incubation period are necessary to produce a quality product. Inoculum production requires colonization of wheat with not less than 10^6 conidia per kg of seed. At this level 100% of seeds are colonized. A minimum of 25% moisture is required during incubation of the wheat seed to ensure sufficient growth of *A. flavus*. A visual examination (using a microscope at x63 magnification) of the surface of fixed, stained wheat seed incubated for 24 h showed that a moisture content less than 25% resulted in scanty growth of the fungus (scored as the quantity of hyphal growth on the seed surface). Incubation period is also critical. For adequate colonization an incubation period of at least 18 h at 31 C was required. Visual examination of wheat grain incubated for different periods showed significantly less surface colonization on seed incubated under 18 h. Colonized seed must subsequently be dried to prevent fungal growth during shipping and storage. Drying also allows the fungus to enter a quiescent state until the product reaches its target area and is reactivated by moisture. This allows conservation of food reserves and maintains product flowability.

The efficacy of the wheat seed formulation has been illustrated in both field plot experiments and pilot studies in commercial settings. In addition, the wheat seed product is straightforward to produce and eliminates cumbersome manufacturing needs required in alginate pellet formulation. Wheat seed is cheap, readily available and already has characteristics (granular and flowable) that are ideal for application using a variety of methods. The tough outer seed coat and dry formulation makes the product stable for long periods and highly resistant to damage. A comparatively small amount of moisture (25% by weight) is required for inoculation, and the process of colonization occurs over a brief period (18 h), after colonization the product can be rapidly dried for packaging and transported to the site of application. Colonization of the wheat seed prior to application reduces the likelihood that the wheat will be exploited by other, unintended organisms.

Wheat seed colonized with atoxigenic *A. flavus* (or a similar inert carrier and food source) may have application on other crops where aflatoxin contamination is a recurrent problem, and may be relevant to aflatoxin control in less developed countries. The extent to which the above wheat seed formulation and manufacturing process are utilized will depend on the economics of production versus the benefits of aflatoxin control.

ANALYSIS OF THE *ASPERGILLUS FLAVUS* POPULATION IN A CALIFORNIA ORCHARD

Sui-Sheng T. Hua, James L. Baker and Melanie Flores-Espiritu. USDA, ARS, Western Regional Research Center, Albany, CA

Aspergillus flavus is a common microorganism in nut-tree orchards. Infection of these edible nuts by the fungus results in aflatoxin contamination of the commodity. The contaminated nuts with aflatoxin level above 20 ppb are considered to be hazardous to human health and would be banned from the market and rejected by importing countries. It is well established that *Aspergillus flavus* can be subdivided into two groups, S and L. Isolates of S strain consistently produce large amount of aflatoxin and sclerotia, while L strain does not produce sclerotia and has variable content of aflatoxin. Analysis of the population of this obnoxious fungus in tree-nut orchards may provide useful information in developing strategies for effective biological control. Air samples were collected during the year of 1998 from March to September in Wolfskill Grant Experimental Farm in Winters, CA.. Buds and flowers of almonds and pistachios were collected in spring during the month of March and April. Leaves , stems, immature fruits and mature fruits were sampled each month from May to September. A special agar medium was used to facilitate the isolation of *A. flavus* and coconut agar was used to screen for aflatoxin-producing isolates. Preliminary results indicate that some buds and flowers of almonds and pistachios were infected by *Aspergillus flavus* strains even though the temperatures were mild in those months and the air samples did not show the presence of this fungus. These results suggest that flowers might provide an entry for infecting the kernels. *A. flavus* was found on leaves, stems and fruits during the growing season and the number of pistachios fruits contained *A. flavus* remained to be 5-7% of the total nuts analyzed. *A. flavus* was detected in the air sample in the month of July, August and September. Out of 115 isolates , 41 strains formed sclerotia on PDA agar and 74 isolates are L strain. Only a few isolates of L strains produced aflatoxin. Therefore applying additional nontoxigenic strain of *A. flavus* to the orchards for competing off the toxigenic strains may not be able to solve the aflatoxin contamination problem. Alternative antagonistic microorganisms should be searched for to reduce the population of *A. flavus* , particularly the toxigenic strain. Long term monitoring of the population of *A. flavus* may provide important insight regarding whether the cultivation of these two tree-nut crops enriches the toxigenic strains.

CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS

PLATFORM PRESENTATIONS

PANEL DISUCSION

Panel Discussion Title: Crop Management and Handling, Insect Control and Fungal Relationships

Panel Members: Michael Hurley (Chair)

Summary of Presentations: P. Dowd (NCAUR) reported on 1998 tests of the effectiveness of the Bt corn gene in suppressing European corn borer and fumonisin levels. In those hybrids where Bt was expressed strongly, both insect and toxin levels were greatly reduced, in others only the insect levels showed reduction. Toxin reduction appeared to depend on the absence of competing insect populations, particularly sap beetle and corn earworms. Research is continuing on the effect of anionic peroxidase on insect resistance in model plant systems.

M. Doster (UCD, Kearney Ag. Center) reported on the effect of rootstock and of Volck oil spraying on early split formation in pistachios. Trees on PGII rootstock showed roughly a doubling of early splits compared with trees on other rootstocks. Similar increases were seen after spraying Volck oil in the spring and the effects were additive. In another experiment, fungal decay more than tripled, and navel orangeworm infestation increased sixfold from the first harvest to the last 20 days later. Furthermore, early splits continued to form after the first harvest. P. Cotty asked about the increase in early splits between harvests. M.Doster said that typically early splits continue to form if harvest is delayed, although usually not as much as in the orchard observed. A possible cause is the uneven maturing of the nuts, which can be caused by inadequate winter chilling. T. Schatzki pointed out that early splits that occurred 2-6 weeks before standard harvest dates are the cause of high aflatoxin levels, while early splits that occurred during the last 2 weeks before harvest have low aflatoxin levels. Doster added that for a late harvest, the early splits with high aflatoxin levels would probably occur during a longer period, for example, 2-8 weeks before harvest. Another question concerned the effect of delaying harvest on yield. Doster responded that yield was not measured, but if harvests are delayed too long then mature nuts will fall from the tree before they are harvested. Doster also mentioned the work with Pearson (WRRC) who successfully expanded the algorithms for the newly developed image sorter to recognize certain shell characteristics (such as insect-caused oily shell, dark stains, and adhering hull) that are characteristic for nuts with kernel decay. H.Lee of HLK Farms asked Pearson whether the image sorter had been tested on corn. Pearson responded that preliminary tests looked promising.

Schatzki (WRRC) reported improved calculations in predicting sampling variance as applied to the new EU aflatoxin standard for pistachios. Algorithms for insect detection, based on film x-ray images have become successful, but because of the success of the image sorter (see above) will probably not be implemented commercially. Successful algorithms for pinhole (Indian meal moth) detection in almonds have been developed, also based on film

x-rays; they but will need to be expanded to high speed, high resolution x-ray imaging. Such a system is under development. The aflatoxin distribution in NOW damaged almonds has been measured and found to differ from that found previously in all pistachio populations, presumably because early splitting plays no role in almonds. Early results show promise to develop a high-speed, non-destructive test for high aflatoxin levels in peanuts. Such a test is required to develop a sorter for reducing aflatoxin in shelled peanuts.

Doster reported that while first crop Conadria figs showed more decay from *A.flavus* than main crop figs, they did not have more aflatoxin. Breeding programs have successfully reduced the ostiole size in Calimyrna-type figs with a concurrent reduction of insect damage as insects, can no longer enter. Collection of propagules on leaves and in soil 3 weeks before harvest showed correlation between propagule densities and fruit decay.

Batson (MissSU) reported that increases in aflatoxin levels have appeared to coincide with the increase of the use of cotton modules over the last ten or more years. Accordingly, modules were constructed and placed in Arizona and Mississippi with testing ports for sampling over time. Fields had been seeded with toxigenic fungi. Arizona aflatoxin results were found to be quite variable and no conclusions could be drawn. Little aflatoxin was noted in Mississippi.

The audience and panels were asked whether the research being carried out could have prevented the 1998 aflatoxin outbreak throughout the Southern US. Cotty (SRRC) pointed out that the introduction of non-toxigenic fungi would probably not be economically viable in the South, although it is economic in the more consistently hotter and dryer Arizona. Schatzki noted that post-harvest research does not address aflatoxin production per se, although it aims to reduce the levels entering the food stream.

ADVANCES IN INSECT-ORIENTED IPM OF MYCOTOXIGENIC FUNGI IN CORN IN THE MIDWEST - FY 1998

Patrick F. Dowd,[1] Robert J. Bartelt,[1] Robert W. Behle,[1] Michael R. McGuire,[1] L. Mark Lagrimini,[2] Juan J. Estruch,[3] David A. Kendra,[3] Martha Hill,[3] Laura S. Privalle;[3] Martha Wright,[3] Gary Molid,[4] Ivan Haase;[5] Johnathan P. Duvick.[6] [1]USDA-ARS, NCAUR, BAR, Peoria, IL; [2]Ohio State University, Columbus, OH; [3]Novartis Seeds, Inc., Stanton MN and Research Triangle Park, NC; [4]Del Monte, Manito, IL; [5]Bridgeway, Macomb, IL; [6]Pioneer Hi-Bred, Johnston, IA

Insect Resistance - Total fumonisin levels in ears sampled in 1997 from 1-2 acre fields near Kilbourne IL of NK6800Bt and Pioneer 33V08 were from ca. 30-40 times lower than corresponding nonBt hybrids in 1997 (1.4 vs. 0.049 ppm and 1.1 vs. 0.028 ppm, respectively). Total fumonisin levels in small plot studies at Peoria in 1997 for NK6800Bt were ca. 8 times lower that those from the corresponding nonBt hybrid (the Pioneer series was not tested). These hybrids express the Bt gene at high levels throughout the plant (including ears and silks), and had negligible caterpillar damage in 1997, compared to corresponding hybrids that had up to 91% infestation of European corn borer. Corn earworm presence in all hybrids was limited. Total fumonisin levels in the MAX454 Bt hybrid (low Bt protein expression in silks and kernels) were not significantly different from those of the corresponding nonBt hybrid, at Kilbourne or Peoria in 1997, despite significantly lower levels of infestation by European corn borer (as reported last year). In 1998, corn earworms were generally more common than European corn borers at experimental fields and commercial fields, although corn earworm infection ranged from 6% to 40% in the same hybrid expressing the Bt gene throughout the plant, depending on the location. Although incidence of caterpillar damage was often significantly lower in the Bt vs. nonBt hybrids, the degree of difference in incidence and numbers of damaged kernels were not nearly as great as those seen in 1997. This is in contrast to other reports that indicated the same Bt hybrids expressing the genes throughout the plant (Mon810 and Bt11 events) produced "nearly complete control" of corn earworms (American Entomologist 44:75). Sap beetle incidence was also much higher in 1998 than 1997 (up to 40% infestation at milk stage). These high rates of sap beetle infestation were presumably due to the mild winter that allowed higher survival rates as indicated by early spring trap comparisons for 1997 vs. 1998. There were also fewer significant differences in incidence of ears with signs of Fusarium infestation in 1998 for the hybrids expressing the Bt gene at high levels throughout the plant (2.5 vs. 0% and 10.5 vs. 2%) (mycotoxin analyses pending). Based on studies with Bt corn dating back to 1995, it appears that a substantial reduction in mycotoxigenic fungi and mycotoxins can occur when the target insect (European corn borer) is present, even at high numbers. When other insects are present, especially those that do lots of damage, such as corn earworms, reductions in mycotoxins are more limited or nonexistent, even for Bt hybrids that express the gene throughout the plants. Newer Bt genes that produce proteins relatively more toxic to corn earworms

should help lower corn earworm incidence and associated mycotoxigenic molds and mycotoxins.

Tobacco and tomato transformants expressing high levels of tobacco anionic peroxidase showed significantly enhanced resistance to feeding by tobacco hornworms, as was noted previously for corn earworms. When dose consumed was considered, there were no significant differences in susceptibility to nuclear polyhedrosis virus of corn earworms fed wild type or transformed tobacco or tomato plants expressing high levels of tobacco anionic peroxidase. Some initial transformants of corn expressing tobacco anionic peroxidase killed 100% of European corn borers and corn earworms tested, and significantly reduced growth rates of fall armyworms. Progeny of these transformants were negligibly affected by high levels of European corn borers (the only insect species tested), while corresponding untransformed plants were destroyed. Work continues on the role of peroxidases in disease resistance.

Monitoring and Prediction - Modified sap beetle traps/autoinoculators were produced by a commercial company as a test run. In order to increase the potential market for the traps, they were tested in Bt sweet corn grown by Del Monte and compared with conventional sampling techniques for sap beetles at a research site established by the two county Central Illinois Irrigated Growers Association. Attractants remained relatively effective over a two week period, and traps captured readily quantifiable numbers of sap beetles. Trap captures generally reflected incidence of beetles on corn ears, and ear infestation, but determinations of sap beetle presence were much more rapid using traps vs. standard sampling techniques of nondestructive individual ear inspection. Although the Bt hybrid had very limited damage by corn earworms compared to the nonBt hybrid (11% vs. 81%, respectively), sap beetle incidence was similar (31% vs. 40%, respectively). Sap beetle damage was most severe in ears where husk coverage did not extend beyond the ear tip.

A predictive program for aflatoxin and fumonisin mycotoxin incidence in midwest corn has been written for use on IBM compatible microcomputers. It involves two points of prediction: one prior to pollination so that growers can apply insect or disease control measures during ear fill if desired, and the second for harvest time prediction so early harvest can be performed to reduce ultimate levels of mycotoxins if desired. Input types were designed based on consultations with local growers. The program can store known information about hybrids that includes insect and fungal resistance, or accept direct input. Temperature and rainfall/irrigation water and soil type are included as variables as well. The program is still being debugged and predictive algorithms are being fine-tuned. Better sources of past data will continue to be used to evaluate the program and to further adjust the predictive algorithm. Aesthetic improvements are also needed.

Biological control - An additional site that the nematode parasite of sap beetles can be collected from was discovered in Illinois in Mason Co. Several hundred sap beetles

presumably infected with this nematode were released in an agricultural area that the nematode does not occur.

The insect pathogen *Beauveria bassiana* was again released in autoinoculators baited with attractants for sap beetles. In some cases, relatively high rates of infections (100 % of some sap beetle species) were recovered from overwintering traps. Although up to 20% on average of a particular sample date of sap beetles collected in traps set out in the spring were infected with *B. bassiana*, these levels were lower than those encountered in 1997. This difference is presumably due to higher levels of individual sap beetles than normal successfully overwintering in the ground during the mild winter, which would be less likely to be infected than those that overwinter in groups in tree holes.

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AFLATOXIN CONTROL IN PISTACHIOS

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The incidence of fungal decay in early splits harvested at three different dates in 1997 increased only slightly from 85% for the first harvest to 96% for the last harvest 20 days later, while the incidence in nuts with cracked hulls increased substantially from 26 to 70%. For all types of nuts considered together, fungal decay more than tripled and navel orangeworm infestation increased sixfold from the first harvest to the last. Nuts with shriveled hulls had substantially higher incidences of decayed and insect-infested kernels than nuts with smooth hulls. A similar experiment was performed in 1998, and the nut samples are currently being evaluated.

Studies with chemicals that induce pistachio trees to break dormancy early were continued in 1998. A single application of Volck oil sprayed in mid-January (5.0% early splits at harvest time), in mid-February (5.8%), or in mid-March (5.2%) increased the formation of early splits compared to the nonsprayed control (2.8%). Even one month before harvest substantially more early splits occurred on trees sprayed with Volck oil (between 0.8 and 1.3% depending on application date) than on nonsprayed trees (0.1%).

The type of rootstock used affected early split formation. Trees on PGII rootstock had a substantially higher (5.5%) incidence of early split nuts at harvest than trees on PGI (3.3%), *Pistacia atlantica* (2.9%), or UCB (2.6%). Likewise, for trees sprayed with Volck oil, trees on PGII had a substantially higher (6.8%) incidence of early splits than trees on *P. atlantica* (3.9%) or UCB (3.0%).

Sorting algorithms for an image sorter were developed for several shell defects that are characteristic of early split or navel orangeworm-infested nuts. The image sorter was adapted to distinguish nuts with oily stains, dark stains, and adhering hull from normal nuts with a validation set false positive rate of 1.4% and a false negative rate of 2.3%.

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POST-HARVEST REMOVAL OF AFLATOXIN FROM TREE AND GROUND NUTS

Thomas C. Pearson, Soo Won Kim, Ronald P. Haff, Martin S. Ong, and Thomas F. Schatzki and. USDA, ARS, Western Regional Research Center, Albany, CA

1. Pistachios - (a) Real time image sorter. USP 5,703,784, Dec. 1997, *license in negotiation*, installation Oct. 1998 *Sorting for other quality defects.* J. Agric. Food Chem 46:2248-2252 (1998); (b) Sampling. Mean and variance of samples for acceptance of lots. Normal distributions: J. Agric. Food Chem 46:2-4 (1998). Actual distributions, Monte Carlo calculations, buyer and seller's risk: submitted for clearance for *J. Agric. Food Chem.* For 98% acceptance requires 0.1 ppb lots, regardless of sample size. Impact on EU market; (c) Insect detection by x-ray. New classification algorithm (automatic quadratic combinations of histogram features on 17-nearest neighbor) scanned-film images yields 65% of infested nuts detected with 1% false positives, a commercially viable point. *Proc. SPIE 3545-14 (1998)*

2. Almonds - (a) Insect (Indian Meal Moth) pinhole detection by x-ray. Detection by intensity and derivatives of images. Removal of germ region by localization. In scanned-film images 85% recognition with 1% false positives. In line-scanned images 65% recognition. 9% false positives. For *Trans. ASAE. Image intensifier images to be tested;* (b). NOW damaged almonds. Distribution function of aflatoxin in single nuts shows smooth curve (no minimum)for c=100-1.000.000 ppb with p= $3x10^{-4}$ - $2x10^{-5}$, as opposed to pistachios. All nuts show aflatoxin at 0.03-0.3 ppb. For *J. Agric. Food Chem.*

3. Walnuts - *The* ***single nut distribution*** *of aflatoxin in an insect infested early cultivar (Serr) walnuts will be measured to test the effect of early infestation on the shape of the distribution.*

4. Peanuts - (a) *Attempts will be made to develop a* ***better sorter for aflatoxin*** *in peanuts. As a first step, a method will be developed to non-destructively select peanuts which contain 10.000 ppb or more of aflatoxin.* Attempts to use solid-state NMR failed due to lack of sensitivity. MS-MS of extraction fluid in which individual peanuts were rinsed shows promise. *Such selected peanuts will be inspected for characteristics which could be used for sorting;* (b) *The* ***single nut distribution*** *of aflatoxin in peanuts will be measured to develop sampling statistics, develop sorting targets and to test the negative binomial distribution.*

Italics indicate future work.

AFLATOXIN CONTROL IN FIGS

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Although first-crop Conadria figs from the 1997 harvest in three commercial orchards had substantially more fungal decay than main-crop figs, first-crop figs had approximately the same incidence of decay caused by fungi in *Aspergillus* sect. *Flavi* and the same levels of aflatoxin contamination as main-crop figs. First-crop figs, however, had three times more colonies on the fig exterior than main-crop figs. During the 1998 harvest, samples of first-crop figs were collected from two Conadria orchards (while the main-crop was maturing and again during the main-crop harvest) and are in the process of being evaluated.

The effect of burying drip irrigation lines on fungal decay of figs was investigated. Samples of soil, leaves, and fruit have been collected from trees irrigated by surface drip irrigation and from those irrigated by buried or subsurface drip. These samples are being evaluated.

The fruit harvested in 1997 from thirteen new Calimyrna-type fig cultivars were evaluated. The mean eye (ostiole) diameter ranged from 0.8 to 2.0 mm for these new cultivars, which is substantially smaller than the typical eye size for Calimyrna figs. As the mean eye diameter decreased, the incidence of insect damage to the fruit also decreased. No decay by aflatoxigenic fungi was observed, although other fungi in *Aspergillus* decayed 1.3% of the figs from these selections.

The relationships between various factors (such as densities of propagules in the soil or on leaves) in July and the incidence of fungal decay by aflatoxin-producing fungi at harvest (21 August) were determined for ten commercial orchards in 1997. The second collection date of leaves and soil (31 July) provided better prediction of fungal decay of the fruit than the first collection date (1 July). For the second collection date, the levels of propagules in the soil and on leaves were positively correlated with fruit decay caused by *Aspergillus* sect. *Flavi*. Additional samples have been collected in 1998 and are being evaluated.

Publications:

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AFLATOXIN CONTAMINATION AT WEEKLY INTERVALS IN COTTON MODULES CONSTRUCTED IN ARIZONA AND MISSISSIPPI

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Two cotton modules, MS1 and MS2, were constructed at the Plant Sciences Research Center at Starkville, MS on October 1 and 2, respectively, from fields infested at boll-crack with 2.8 lb./A of wheat kernels colonized with a highly toxigenic isolate of *A. flavus*. These modules remained in the field throughout the study. In addition, two modules were constructed at Texas Hill Farms near Roll, Arizona from fields in an area with a history of aflatoxin problems. The Arizona modules, AZ1 and AZ2, were constructed on October 9, 1997. Arizona modules were transported to the gin-yard for storage and sampling. Module sampling ports (MSP) were installed within all modules during construction to permit access to the module interior for repeated sampling. Seed cotton samples were taken from each sampling port at module construction and weekly for five weeks for the Arizona and Mississippi modules. Also, two samples were collected weekly from the exterior of each module. Samples from Arizona were shipped overnight to Mississippi for analysis. Arizona and Mississippi samples were ginned , inspected for seed fluorescence and subsamples analyzed for Aspergillus seed infection, free fatty acids, and aflatoxin contamination.

Aflatoxin levels in seed from individual sampling ports of Arizona modules were extremely variable and ranged from 0 to 920 and 0 to 3500 ppb for modules AZ1 and AZ2, respectively. Of 175 possible sampling events (sampling sites x sampling periods x modules), aflatoxin was detected in 31% of the Arizona samples. Where detected, aflatoxin was above 20 ppb in 46% of the samples. Aflatoxin was detected from composite samples composed of residual cottonseed meal from all sampling ports of AZ1, or AZ2 for all sampling periods. Maximum aflatoxin concentration detected during the sampling period in composite samples was 123 and 447 ppb for AZ1 and AZ2, respectively. There was no indication that aflatoxin concentration increased over time with storage in the 1997 Arizona modules. Mean percentages of fluorescent seed, Aspergillus seed infection and free fatty acids were 0.04, 1.2 and 0.46 and 0.13, 1.5 and 0.5 for AZ1 and AZ2, respectively.

Aflatoxin was detected in only 6% of the sampling events from the Mississippi modules. Where detected, aflatoxin exceeded 20 ppb in only one case. Mean percentages of fluorescent seed, Aspergillus seed infection and free fatty acids were 0.06, 0.23 and 0.6 and 0.05, 0.02 and 0.7 for MS1 and MS2, respectively. There was a significant increase in % free fatty acids in seed from MS2 over time.

POSTER PRESENATATIONS

OXIDATION AND DETOXIFICATION OF AFLATOXIN B1 IN CORN: APPLICATION OF ELECTROCHEMICALLY GENERATED HIGH CONCENTRATION OZONE

K. Scott McKenzie,[1] Brian N. Findeisen,[1] Adrian J. Denvir,[1] G. Duncan Hitchens,[1] Timothy D. Phillips,[2] Leon F. Kubena[3] and Tom D. Rogers.[1] [1]Lynntech, Inc., College Station, TX; [2]Texas A&M University, College Station, TX; [3]USDA/ARS Food Animal Protection, College Station, TX

Previous studies have demonstrated that a novel, electrochemical source of ozone gas (O_3) may be used to chemically degrade numerous mycotoxins, including aflatoxin B_1 (AfB_1). I*n vitro* toxicological analyses demonstrated the successful detoxification of AfB_1, suggesting a new approach to remediation aflatoxin contaminated grain. Further studies demonstrated that 30 kg of aflatoxin-contaminated whole kernel corn could be treated with 14 wt% (140.000 ppm) ozone gas at 200 mg/min to effectively reduce the concentration of AfB_1 from 1.220±73.3 ppb AfB_1 to 58.4 ± 18.2 ppb, a reduction of over 95% in 92h. Subsequent *in vivo* analysis demonstrated turkeys fed AfB_1 corn had reduced body weight gain and relative liver weight, whereas turkeys fed control corn + O_3 or AfB_1 corn + O_3 did not differ from controls. Furthermore, alterations in the majority of relative organ weights, liver discoloration, serum enzyme activity, hematological parameters, and blood chemistry caused by AfB_1 were eliminated (no difference from controls) by treatment with O_3. It is important to note that treatment of control corn with O_3 did not alter the performance of the turkey poults. Proximal analysis demonstrated that ozone treated corn did not affect crude fat, protein, fiber or ash, further suggesting that levels of ozone necessary to detoxify corn may not significantly affect the nutritional or palatability character of the corn. While treatment of contaminated corn with electrochemical O_3 appears to be a viable method for detoxification and fungal decontamination of devalued grain, it should be noted that ozone source and concentration, contacting of the ozone with the aflatoxin molecule and grain condition are important factors affecting efficiency of aflatoxin degradation.

REGULATION OF AFLATOXIN BIOSYNTHESIS

POSTER PRESENTATIONS

AFLATOXIN PRODUCTION BY *ASPERGILLUS* SPECIES OTHER THAN *A. FLAVUS* AND *A. PARASITICUS*

M. A. Klich, E. J Mullaney and J. W. Cary. USDA, ARS, Sourthern Regional Research Center, New Orleans LA

DNA of aflatoxin-producing strains of *Aspergillus tamarii (A. caelatus), A. nomius* and *A. ochraceoroseus* was examined by Southern hybridization for similarities to known aflatoxin and sterigmatocystin biosynthesis genes from *A. flavus/parasiticus* and *A. nidulans*. Under the high stringency conditions used throughout this study, the *A. tamarii* and *A. nomius* DNA hybridized to all four of the *A. flavus/parasiticus* gene probes. This indicates that there are strong similarities in the biosynthetic pathway genes of these species and those of *A. flavus* and *A. parasiticus*. These four species all belong to the same group - *Aspergillus* section *Flavi*.

In earlier studies, other *A. tamarii* isolates did not hybridize to the aflatoxin biosynthetic pathway genes from *A. flavus/parasiticus*, indicating that the isolate examined in this study is unusual. The strong similarity of the genes of the aflatoxin-producing *A. tamarii* to those of the *A. flavus/parasiticus* biosynthetic pathway genes indicated by our data from Southern hybridizations and the apparent absence of these genes in other isolates of this species raises the possiblility that this *A. tamarii* isolate acquired aflatoxigenicity via horizontal transfer from *A. flavus* or *A. parasiticus*.

Aspergillus ochraceoroseus hybridized to the *A. nidulans afl*R probe and weakly to the *A. nidulans stc*L and *A. flavus ver*B probes, but did not hybridize with any of the other sterigmatocystin or aflatoxin biosynthetic pathway gene probes. This species produces both aflatoxin and sterigmatocystin and belongs to a different section of the genus (section *Circumdati*) from the other aflatoxin- and sterigmatocystin-producing species. This is the first species outside of *Aspergillus* section *Flavi* reported to consistently produce high levels of aflatoxin, and the only isolate we know of that produces both aflatoxin and sterigmatocystin. Although researchers in the Ivory Coast have reported repeated isolation of this species, only one isolate has been made available to other researchers, so comparison of different strains of this fungus is impossible. Our data indicate that at the DNA level, the aflatoxin and sterigmatocystin biosynthetic pathway genes for this species are somewhat different from those of the known pathway genes.

IS THE *AFLT* GENE PRODUCT, A TRANSMEMBRANE PROTEIN, INVOLVED IN AFLATOXIN SECRETION?

Perng-Kuang Chang, Jiujiang Yu, Deepak Bhatnagar, and Thomas E. Cleveland. USDA, ARS, Southern Regional Research Center, New Orleans, LA

Most of the genes that are involved in aflatoxin biosynthesis as well as in sterigmatocystin biosynthesis have been found to be clustered. Adjacent to *pksA*, which encodes a polyketide synthase, a gene named *aflT* was identified by chromosomal walking. The *aflT* gene was transcribed opposite to the orientation of *pksA* and contained five introns. The predicted AFLT protein was a transmembrane protein with 14 membrane associated helices. AFLT was homologous to the *Cochliobolus carbonum* HC-toxin efflux pump protein and to many multidrug-efflux transporter proteins. The *aflT* gene was found in aflatoxigenic *A. parasiticus* SU-1 as well as in OMST-accumulating SRRC 2043. The location and the predicted protein structure of *aflT* suggest that it is the aflatoxin transporter gene.

GENETIC LINKS OF AFLATOXIN PRODUCTION AND HEXOSE UPTAKE

Jiujiang Yu, Perng-Kuang Chang, Deepak Bhatnagar, and Thomas E. Cleveland. USDA, ARS, Southern Regional Research Center, New Orleans, LA

Aflatoxins are known as potent carcinogens and pose a serious threat to the health of human beings. Understanding of the biological and genetic aspects of aflatoxin production biologically and genetically are the key to the elimination of aflatoxin contamination in food and feed. It is known that nutritional factors directly or indirectly control the aflatoxin production. A sucrose or glucose medium supports aflatoxin production in aflatoxigenic fungi, while peptone (or complex sugar) medium represses aflatoxin production. Several new genes have been recently cloned on the aflatoxin pathway gene cluster. A gene, named *hxtA*, shares over 30% amino acid identity with the hexose transporter protein in yeast and other organisms. Hydrophobicity plot indicated that this protein contains 12 membrane spanning regions responsible for high-affinity and low-affinity 6-carbon-sugar uptake such as glucose and galactose. Northern blot analysis demonstrated that the timing of the expression of this *hxtA* gene is the same as those of the aflatoxin pathway genes in PMS/A & M media. A gene coding for alpha 1-6 glucosidase has been also cloned next to the *hxtA* gene. This gene is also expressed in aflatoxin conducive medium. Since these two genes are located along with the aflatoxin biosynthetic pathway gene cluster, and are not expressed constitutively, we can reasonably assume that the hexose utilization and the aflatoxin biosynthetic pathway are genetically and biologically linked.

COMPLEMENTATION OF AN AVERUFIN-ACCUMULATING MUTANT OF *ASPERGILLUS PARASITICUS*

Charles P. Woloshuk[1] and Jiujiang Yu.[2] [1] Purdue University, West Lafayette, IN; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus*. Genes involved in the biosynthesis of aflatoxins are clustered in the fungal genome. While most of the DNA sequence is known, the function of several genes in the cluster remains to be elucidated. We transformed a mutant of *A. flavus* lacking the entire aflatoxin gene cluster with two overlapping cosmids (5E6 and 8B9) and recovered transformants that accumulated the aflatoxin pathway intermediate averufin (AVR). The addition of a third cosmid (13B9) restored aflatoxin biosynthesis, suggesting that the gene, *avfl*, involved in the conversion of AVR to versiconal acetate (VA) is located on cosmid 13B9. The *A. parasiticus* strain ATCC 24551 that was isolated by Donkersloot in 1972 also accumulates AVR because of an apparent block in the conversion of AVR to VA. This mutation was complemented with cosmid 13B9. Transformants containing cosmid 13B9 produced wild-type levels of aflatoxin. These data suggest that strain ATCC 24551 contains a mutation in *avfl*.

IDENTIFICATION OF THE DNA BINDING SITE FOR THE *ASPERGILLUS FLAVUS* AFLR IN THE NOR-1 PROMOTER

Carmen S. Brown-Jenco,[1] Greg R. OBrian,[2] Sarah Sloan,[2] and Gary A. Payne.[2] [1] Monsanto, St. Louis, MO; [2]North Carolina State University, Raleigh, NC

Aflatoxins are secondary metabolites produced during idiophase by at least four species of *Aspergillus*. Induction of aflatoxin biosynthesis appears to involve the interplay of transcriptional regulatory elements and physiological factors that affect fungal metabolism. The global transcriptional regulator for aflatoxin biosynthesis is AflR. This protein has been classified as a Zn(II)2Cys6 regulatory protein. Here we report the use of a GUS reporter system to locate a region between 91 and 138 bp upstream of the translational start site of *nor-1* that is required for AlfR regulation of *nor-1* transcription. By the use of Southwestern analysis, we further show that a purified heterologous form of AflR specifically binds to an oligo containing this site. The consensus binding site found is TCGgccagCGA, which is similar to that reported for the *A. nidulans aflR* and the *stcU* promoter.

CHARACTERIZATION OF AFLJ AND ITS PUTATIVE ROLE IN AFLATOXIN BIOSYNTHESIS

Wanglei Du,[1] Greg R. Obrian,[1] Deepak Bhatnagar,[2] and Gary A. Payne.[1] [1]North Carolina State University; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA

Aflatoxin biosynthetic pathway genes are clustered in a 75-kb DNA fragment. *aflJ* resides in the cluster adjacent to the pathway regulatory gene *aflR*. Both genes are required for aflatoxin production and the conversion of exogenously added pathway intermediates to aflatoxin, but *aflJ* is not required for the transcription of the pathway genes. Because these two genes are divergently transcribed from a 737-bp intergenic region, we have been interested in their regulation. The objective of this study was to determine if *aflR* is required for the expression of *aflJ*. Two gene constructs were used to test the effects of *aflR* on *aflJ* gene expression. One construct, pGAP23, contains a constitutive promoter *gpdA* fused with the *aflR* open reading frame, and the other construct, pGAP28, has a β-glucuronidase reporter gene driven by the *aflJ* promoter. *Aspergillus flavus* strain 649-1, which lacks the aflatoxin biosynthetic cluster, and the aflatoxin producing strain 86-10 were transformed with these gene constructs. Overexpression of *aflR* in 86-10 containing *aflJ*::GUS showed constitutively expressed *aflJ* and elevated GUS activity even under conditions nonconducive for aflatoxin production. Transformants of 649-1 containing pGAP28 alone produced background levels of GUS. Transformants containing both pGAP23 and pGAP28 resulted in increased GUS activity on aflatoxin conducive media. From these studies, we conclude that *aflR* is required for the transcription of *aflJ*, and because 649-1 lacks the cluster, no other genes in the cluster are required. The binding site for *AflR* in the *aflJ* promoter is not known.

MODIFICATION OF THE MUTAGENIC POTENTIAL OF AFLATOXIN BY INTRINSIC COMPONENTS OF CORN AND COTTONSEED, AND BY FUMONISIN

Douglas L. Park, Armando Burgos-Hernandez, Henry Njapan and Rebeca Lopez-Garcia.
Louisiana State University, Baton Rouge, LA

Increased scientific knowledge and the establishment of regulation have reduced but not eliminated exposure to aflatoxins. Complete elimination is uneconomical for producers and may deprive consumers of sources of nourishment. Consumption of substances that offset the deleterious effects of aflatoxins, particularly those intrinsic to a commonly consumed foodstuff circumvent the problem. This study employed a bioassay directed fractionation using the *Salmonella*/microsomal mutagenicity assay (tester strains TA100 and TA98), and TLC to isolate and study those constituents of corn and cottonseed with a potential to inhibit the mutagenicity of aflatoxin. Results obtained to date show that corn and cottonseed contain several compounds with varying degrees of anti-mutagenic activity, among them linoleic acid. This study has, therefore, demonstrated the potential of corn and cottonseed as sources of anti-mutagenic substances that could be exploited to benefit the health of consumers and revenue of producers.

INABILITY OF AFLATOXIN BIOSYNTHESIS IN KOJI MOLD (*ASPERGILLUS SOJAE*)

Kenichiro Matsushima,[1] Kumiko Yashiro,[1] Yoshiki Hanya,[1] Keietsu Abe,[1] Kimiko Yabe,[2] and Takashi Hamasaki.[3] [1] Kikkoman R&D Center, Noda City, Japan; [2]National Food Research Institute, Tsukuba, Japan; [3]Tottori University, Tottori, Japan

Ten *A. sojae* strains used for soy sauce production were chosen for analysis of growth on bleomycin medium and aflatoxin production. *A. sojae koji* molds used for soy sauce production tested in this study were distinguished from *A. parasiticus* morphologically and/or physiologically. Colony color in age and bleomycin test also showed that all these strains could be classified as *A. sojae*, and no aflatoxin was detected by HPLC analysis of culture extracts from all strains. No detectable transcripts of *aflR*, the main transcriptional regulatory gene for aflatoxin biosynthesis, were observed in these strains under aflatoxin inducing condition for *A. parasiticus*. Strain 477 was chosen for further molecular analysis. Presence of enzymatic activities which catalyze reactions of aflatoxin biosynthesis from norsolorinic acid to aflatoxin were examined. Most of the known enzyme activities were not detected in strain 477 or, at least, they were insignificant compared to the levels found in *A. parasiticus*. Southern analysis indicated that genomic DNA of strain 477 included all the three aflatoxin biosynthetic pathway genes measured, namely *aflR*, *nor-1*, *pksA*; whereas northern analysis revealed that mRNA of these genes were not transcribed under aflatoxin inducing condition. Though most of these *A. sojae* strains possessed aflatoxin biosynthesis related genes, the lack of aflatoxin related enzymatic activities was probably due to the lack of functionality of the transcriptional regulator, *aflR*. It is assumed that some mutation in *aflR* seriously retards the expression of this gene, or that there are defects in the signal transduction system which induces the expression of *aflR*.

APPENDIX

AGENDA

1998 AFLATOXIN ELIMINATION WORKSHOP
OCTOBER 25-27, 1998
ST. LOUIS, MO

SUNDAY, OCTOBER 25, 1998

5:00-8:00 p.m. **REGISTRATION / POSTER ASSIGNMENTS**

6:00 p.m. **POSTER VIEWING**
(Posters will be available for viewing for duration of the meeting beginning Sunday, October 25th.)

6:00-8:00 p.m. **MIXER**

MONDAY, OCTOBER 26, 1998

7:00 a.m. **REGISTRATION / POSTER ASSIGNMENTS**

8:00 a.m. **WELCOME:** Chris Wehrman, CEO, National Corn Growers Association

INTRODUCTORY REMARKS: Jane F. Robens, USDA/ARS/National Program Staff

SESSION 1: CROP RESISTANCE - CONVENTIONAL BREEDING
Chair: Paul Bertels, National Corn Growers Association

8:15 a.m. **1-a** **"Inheritance of, molecular markers associated with, and breeding for, resistance to *Aspergillus* ear rot and aflatoxin production in corn using Tex6."** Donald G. White, Torbert R. Rocheford, Gnanambal Naidoo, Chandra Paul, Andrew M. Hamblin, and Amy M. Forbes. University of Illinois, Champaign, IL

8:30 a.m. **1-b** **Pyramiding resistance to *Aspergillus flavus* and corn earworm to control preharvest aflatoxin contamination: Genetic mapping and DNA markers linked to resistance traits."** Baozhu Z Guo,[1] Neil W. Widstrom,[1] Rugang Li,[2] Peisheng Cong,[2] Robert E. Lynch,[1] Maurice E. Snook,[3] David M.Wilson,[2] and

Thomas E. Cleveland.[4] [1]USDA, ARS, Insect Biology and Population Management Research Lab., Tifton, GA; [2]University of Georgia, Coastal Experiment Station, Tifton, GA; [3]University of Georgia, Athens, GA; [4]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

8:45 a.m. 1-c **"Maize resistance to *Aspergillus flavus* infection: field evaluations and biochemical characterization of antifungal traits."** Robert Brown. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

9:00 a.m. 1-d **"Update on efforts to develop aflatoxin resistant B73 and A632 germplasm."** Charles Martinson. Iowa State University, Ames, IA.

9:15 a.m. 1-e **"A role for the NOR mutant of *Aspergillus parasiticus* in a breeding program to improve resistance to *A. flavus* infection and aflatoxin contamination of corn."** Neil W. Widstrom[1] and David M. Wilson.[2] [1]USDA, ARS, Insect Biology and Population Management Research Lab., Tifton, GA; [2]University of Georgia, Coastal Experiment Station, Tifton, GA.

9:30 a.m. 1-f **"Utility of aflatoxigenic *Aspergillus flavus* and *A. parasiticus* inoculum used in screening of corn and peanut germplasm for resistance to aflatoxin accumulation."** David M. Wilson,[1] Neil W. Widstrom,[2] C. Corley Holbrook,[2] Mike E. Matheron,[3] M. Elizabeth Will,[1] and Jason H. Brock.[1] [1]University of Georgia, Coastal Plains Experiment Station, Tifton, GA; [2]USDA, ARS, Coastal Plains Experiment Station, Tifton, GA; [3]University of Arizona, Yuma, AZ.

9:45 a.m. 1-g **"Integrated fungal/insect resistance to aflatoxin contamination of almond appears durable in field trials despite differential environmental and genotype by environment responses of individual components."** Thomas M. Gradziel and Abhaya Dandekar, University of California, Davis, CA.

10:00-10:30 a.m. **BREAK**

10:30 a.m. 1-h **"Searching for a silver bullet in a haystack."** Corley C. Holbrook,[1] David M. Wilson,[2] M. E. Matheron,[3] and R. D. Wauchope.[1] [1]USDA, ARS, Coastal Plains Experimental Station,

Tifton, GA; [2]University of Georgia, Coastal Plains Research Station, Tifton, GA; [3]University of Arizona, Yuma, AZ.

10:45-11:30 a.m. **PANEL DISCUSSION**
Panel Chair: Corley C. Holbrook

11:30-1:00 p.m. **LUNCH / POSTER VIEWING**

SESSION 2: POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR AFLATOXIN SYNTHESIS IN CROPS
Chair: Howard Valentine, American Peanut Council

1:00 p.m. 2-a **"Molecular basis for environmental effects on aflatoxin production."** Deepak Bhatnagar. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

1:15 p.m. 2-b **"Inhibition of aflatoxin B_1 production in *Aspergillus flavus* NRRL 3357 by sugars, carotenoids and related compounds."** Robert A. Norton. USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

1:30 p.m. 2-c **"Role of polyunsaturated fatty acids on *Aspergillus* developmental processes."** Ana Calvo and Nancy P. Keller. Texas A&M University, College Station, TX.

1:45 p.m. 2-d **"Volatile and nonvolatile natural products of host-plants and microbes affecting insect pests, *Aspergillus* growth and aflatoxigenesis in tree nuts."** Bruce C. Campbell. USDA, ARS, Western Regional Research Center, Albany, CA.

2:00 p.m. 2-e **"Potential use of native plant compounds for prevention of aflatoxin contamination."** Charles Woloshuk, Purdue University, West Lafayette, IN.

2:15 p.m. 2-f **"Genetic studies on the regulation of aflatoxin accumulation."** Gary A. Payne, North Carolina State University, Raleigh, NC.

2:30-3:00 p.m. **BREAK**

3:00-3:45 p.m. **PANEL DISCUSSION**
Panel Chair: Robert A. Norton

SESSION 3: CROP RESISTANCE - GENETIC ENGINEERING

Chair: Robert Sacher, Hunt-Wesson, Fullerton, CA.

3:45 p.m. **3-a** **"Using antifungal proteins against *Aspergillus*. The old and the new of it."** Raymond Bressan. Purdue University, West Lafayette, IN.

4:00 p.m. **3-b** **"Genetic engineering and breeding of walnuts for control of aflatoxin: Progress and potential."** Gale McGranahan, Abhaya Dandekar, and Patrick Vail. University of California, Davis, CA.

4:15 p.m. **3-c** **"Analysis of transgenic cotton and tobacco expressing antifungal proteins and peptides: Part I: Haloperoxidases."** Kanniah Rajasekaran,[1] Jeffrey W. Cary,[1] Thomas J. Jacks,[1] Thomas E. Cleveland,[1] Kurt Stromberg,[2] and Caryl Chalan.[2] [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [2]University of Southwestern Louisiana, Lafayette, LA.

4:30 p.m. **3-d** **"Analysis of transgenic cotton and tobacco expressing antifungal proteins and peptides: Part II: Lytic peptides."** Jeffrey W. Cary,[1] Kanniah Rajasekaran,[1] Anthony J. DeLucca,[1] Thomas E. Cleveland,[1] Kurt Stromberg,[2] and Caryl Chlan.[2] [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [2]University of Southwestern Louisiana, Lafayette, LA.

4:45 p.m. **3-e** **"Genetic engineering of peanut for reduction of aflatoxin contamination."** Peggy Ozias-Akins,[1] Ravinder Gill,[1] Hongyu Yang,[1] and Robert Lynch.[2] [1]University of Georgia, Coastal Plain Experiment Station, Tifton, GA; [2]USDA, ARS, Insect Biology and Population Management Research Lab., Tifton, GA; [2]University of Georgia, Coastal Experiment Station, Tifton, GA.

5:00 p.m. **3-f** **"Transformation of peanut with the synthetic antimicrobial peptide D5C."** Arthur Weissinger and Lori Urban. North Carolina State University, Raleigh, NC.

5:15 p.m. **3-g** **"Genetic engineering of cotton: focusing on expression of anti-fungal compounds in the seed."** Caryl Chlan,[1] Jeffrey W. Cary,[2] Kanniah Rajesekaran,[2] Robert Brown,[2] and Thomas E. Cleveland.[2] [1]University of Southwestern Louisiana, Lafayette, LA; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

5:30-6:15 p.m. **PANEL DISCUSSION**
Panel Chair: Jeffrey Cary

6:15 p.m. **COMMODITY BREAKOUT SESSIONS**

TUESDAY, OCTOBER 27, 1998

8:00 a.m. **ANNOUNCEMENTS**

SESSION 4: MICROBIAL ECOLOGY
Chair: Phillip J. Wakelyn, National Cotton Council, Washington, D.C.

8:15 a.m. **4-a** **"Increased yield in bolls of greenhouse-grown cotton containing *A. flavus* infected seed."** Maren A. Klich. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

8:30 a.m. **4-b** **"Strategies of biological control of *Aspergillus flavus* to reduce aflatoxin contamination in pistachios and almonds."** Sui-sheng T. Hua. USDA, ARS, Western Regional Research Center, Albany, CA.

8:45 a.m. **4-c** **"Monitoring *Aspergillus flavus* community structure over large areas."** Merritt R. Nelson,[1] Thomas V. Orum,[1] Donna M. Bigelow[1] and Peter J. Cotty.[2] [1]University of Arizona, Tucson, AZ; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

9:00 a.m. **4-d** **"Role of vegetative compatibility in the biological control of aflatoxigenic fungi."** Bruce W. Horn, Ronald L. Greene, and Joe W. Dorner. USDA, ARS, National Peanut Research Laboratory, Dawson, GA.

9:15 a.m. **4-e** **"Effect of multi-year application of non-toxigenic strains of *Aspergillus flavus* and *A. parasiticus* on native soil populations."** Joe W. Dorner and Richard J. Cole. National Peanut Research Laboratory, Dawson, GA.

9:30 a.m. **4-f** **"Improving aflatoxin management with atoxigenic strains: Requirements and obstacles."** Peter J. Cotty. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

9:45 a.m. 4-g **"Development and construction of a facility to manufacture commercially useful quantities of atoxigenic strain inoculum."** Larry Antilla. Arizona Cotton Research and Protection Council, Tempe, AZ.

10:00-10:30 a.m. **BREAK**

10:30-11:15 a.m. **PANEL DISCUSSION**
Panel Chair: Maren Klich

SESSION 5: CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS
Chair: Michael Hurley, DFA of California, Fresno, CA.

11:15 a.m. 5-a **"Apparent benefits and limitations of Bt corn for indirectly reducing mycotoxins in the Midwest."** Patrick F. Dowd. USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

11:30 a.m. 5-b **"Aflatoxin control in pistachios."** Themis J. Michailides,[1] Mark Doster,[1] and Thomas C. Pearson.[2] [1]University of California - Davis, Kearney Agricultural Center, Parlier, CA; [2]USDA, ARS, Western Regional Research Center, Albany, CA.

11:45-1:15 p.m. **LUNCH / POSTER VIEWING**

1:15 a.m. 5-c **"Distribution of aflatoxin in pistachios and almonds."** Thomas F. Shatzki and Thomas C. Pearson. USDA, ARS, Western Regional Research Center, Albany, CA.

1:30 p.m. 5-d **"Aflatoxin control in figs."** Mark Doster and Themis J. Michalilides University of California - Davis, Kearney Agricultural Center, Parlier, CA.

1:45 p.m. 5-e **"Aflatoxin contamination at weekly intervals in cotton modules constructed in Arizona and Mississippi."** William E. Batson and Jacobo C. Caceres. Mississippi State University, Mississippi State, MS.

2:00-2:45 pm **PANEL DISCUSSION**
Panel Chair: Thomas F. Schatzki

2:45-3:00 p.m. **CLOSING REMARKS/ NEXT MEETING:** Jane F. Robens, USDA/ARS/National Program Staff

3:00 p.m. **AFLATOXIN TECHNICAL GROUP MEETING**

A Question for Each Panel Discussion:

"Would the research we are pursuing today have protected corn, cotton or peanut crops from the 1998 aflatoxin outbreak throughout the southern U.S.A. ?"

POSTER PRESENTATIONS

1998 Aflatoxin Elimination Workshop
October 25-27, 1998
St. Louis, MO

Poster Board Number

A. MICROBIAL ECOLOGY

A-1 **"Antifungal metabolites, monorden, monocillin IV and cerebrosides from *Humicola fuscoatra* Traaen NRRL 22980, a mycoparasite of *Aspergillus flavus* sclerotia."** Donald T. Wicklow,[1] Biren K. Joshi,[2] William R. Gamble,[2] James B. Gloer,[2] and Patrick F. Dowd.[1] [1]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL; [2] University of Iowa, Iowa City, IA.

A-2 **"Localization of an *Aspergillus flavus* transformant expressing *Escherichia coli* B-glucuronidase in developing maize ears."** Gary L. Windham,[1] W. Paul Williams,[1] Robert L. Brown,[2] T. Ed Cleveland,[2] and Gary A. Payne.[3] [1]USDA, ARS, Mississippi State, MS; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [3]North Carolina State University, Raleigh, NC.

A-3 **"Aflatoxin-producing ability of *Aspergillus flavus* genotypes (RFLP) from fields in eastern Iowa with aflatoxin contaminated corn at harvest in 1988."** Donald T. Wicklow,[1] Cesaria E. McAlpin,[1] Crystal E. Platis,[1] and Lois Tiffany.[2] [1]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL; [2]Iowa State University, Ames, IA.

A-4 **"Production of a biocontrol agent for control of aflatoxin."** Clive H. Bock and Peter J. Cotty. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

A-5 **"Analysis of the *Aspergillus flavus* population in a California Orchard."** Sui-Sheng T. Hua, James L. Baker, and Melanie Flores-Espiritu. USDA, ARS, Western Regional Research Center, Albany, CA.

B. CROP RESISTANCE--CONVENTIONAL BREEDING

B-1 **"Genetic diversity in the GT-MAS:gk population and possible PCR and RFLP markers linked to resistance to *Aspergillus flavus*."** Rugang Li,[1]

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Baozhu Guo,[2] Neil W.Widstrom,[2] Robert E. Lynch,[2] and Thomas E. Cleveland.[3] [1]University of Georgia, Coastal Experiment Station, Tifton, GA; [2]USDA, ARS, Insect Biology and Population Management Research Lab., Tifton, GA; [3]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

B-2 **"RFLP (restriction fragment length polymorphism) screening to map corn resistance to corn earworm and *Aspergillus flavus*."** Baozhu Guo,[1] Rugang Li,[2] Peisheng Cong,[2] Neil W. Widstrom,[1] Robert E. Lynch,[1] David M. Wilson,[2] and Thomas E. Cleveland.[3] [1]USDA, ARS, Insect Biology and Population Management Research Lab., Tifton, GA; [2]University of Georgia, Coastal Experiment Station, Tifton, GA; [3]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

B-3 **"Characterization of a chromosome region which enhances maysin production in corn silks and reduces earworm growth and ear damage."** Baozhu Guo,[1] Peisheng Cong,[2] Rugang Li,[2] Neil W. Widstrom,[1] Billy R. Wiseman,[2] Maurice E. Snook,[3] and Robert E. Lynch.[1] [1]USDA, ARS, Insect Biology and Population Management Research Lab., Tifton, GA; [2]University of Georgia, Coastal Experiment Station, Tifton, GA; [3]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

B-4 **"Relief of aflatoxin contamination through improved drought tolerance."** Kim K. Franke,[1] Craig C. Kvien,[1] C. Corley Holbrook,[2] and Keith T. Ingram.[3] [1]University of Georgia, Tifton, GA; [2]USDA, ARS, Coastal Plains Research Station, Tifton, GA; [3]University of Georgia, Griffin, GA.

B-5 **"Response of resistant and susceptible maize inbreds to inoculation with transformed *Aspergillus flavus* isolates."** Yanelly Alfaro, Gary L. Windham, W. Paul Williams, and Dawn S. Luthe. USDA, ARS, Mississippi State, MS.

B-6 **"Use of an omtA(p)::GUS reporter construct to evaluate corn for resistance to aflatoxin accumulation."** Carmen S. Brown-Jenco,[1] Robert L. Brown,[2] Deepak Bhatnagar,[2] and Gary A. Payne.[3] [1]Monsanto, St. Louis, MO; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [3]North Carolina State University, Raleigh, NC.

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C. CROP RESISTANCE — GENETIC ENGINEERING

C-1 **"A peanut seed lipoxygenase gene responsive to *Aspergillus* infections."** Gloria Burow,[1] Hal Gardner,[2] and Nancy P. Keller.[1] [1]Texas A&M University, College Station, TX; [2]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

C-2 **"Genetic engineering of cotton: Focusing on expression of anti-fungal compounds in the seed."** Caryl Chlan,[1] Jeffrey W. Cary,[2] Kanniah Rajasekaran,[2] and Thomas E. Cleveland.[2] [1]University of Southwestern Louisiana, Lafayette, LA; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

D. REGULATION OF AFLATOXIN BIOSYNTHESIS

D-1 **"Aflatoxin production by *Aspergillus* species other than *A. flavus* and *A. parasiticus*."** Maren A. Klich, Edward J. Mullaney, and J. W. Cary. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

D-2 **"Is the *aflT* gene product, a transmembrane protein, involved in aflatoxin secretion?"** Perng-Kuang Chang, Jiujiang Yu, Deepak Bhatnagar, and Thomas E. Cleveland. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

D-3 **"Genetic links of aflatoxin production and hexose uptake."** Jiujiang Yu, Perng- Kuang Chang, Deepak Bhatnagar, and Thomas E. Cleveland. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

D-4 **"Complementation of an averufin-accumulating mutant of *Aspergillus parasiticus*."** Charles P. Woloshuk,[1] Jiujiang Yu,[2] and Deepak Bhatnagar.[2] [1]Purdue University, West Lafayette, IN; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

D-5 **"Identification of the DNA binding site for the *Aspergillus flavus* AflR in the nor-1 promoter."** Carmen S. Brown-Jenco,[1] Greg R. OBrian,[2] Sarah Sloan,[2] and Gary A. Payne.[2] [1]Monsanto, St. Louis, MO; [2]North Carolina State University, Raleigh, NC.

D-6 **"Characterization of aflJ and its putative role in aflatoxin biosynthesis."** Wanglei Du,[1] Greg R. OBrian,[1] Deepak Bhatnagar,[2] and Gary A. Payne.[1]

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North Carolina State University; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

D-7 **"Modification of the mutagenic potential of aflatoxin by fumonisin and intrinsic components of corn and cottonseed."** Douglas L.-Park, Rebeca Lopez-Garcia, Armando Burgos-Hernandez and Henry Njapan. Louisiana State University, Baton Rouge, LA.

D-8 **"Inability of aflatoxin biosynthesis in koji mold (*Aspergillus sojae*)."** Kenichiro Matsushima,[1] Kumiko Yashiro,[1] Yoshiki Hanya,[1] Keietsu Abe,[1] Takashi Hamasaki,[2] and Kimiko Yabe.[3] [1]Kikkoman R&D Center, Noda City, Japan; [2]Tottori University, Tottori, Japan; [3]National Food Research Institute, Tsukuba, Japan.

E. POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR REGULATION OF AFLATOXIN BIOSYNTHESIS IN CROPS

E-1 **"Purification of a small peptide with antifungal activity against *Aspergillus flavus*."** Anne-laure Moyne, Thomas E. Cleveland, and Sadik Tuzun. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

E-2 **"Contribution of maize kernel constitutive and induced proteins to resistance against *Aspergillus flavus* infection."** Zhi-Yuan Chen. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

E-3 **"New plant compounds inhibitory to aflatoxin production."** Luis Velasquez, Haixin Xu, Seanna Annis, Ray Hammerschmidt, John Linz, and Frances Trail. Michigan State University, East Lansing, MI.

E-4 **"Fungal infection of peanut pods by *Aspergillus parasiticus* during the pre- harvest period."** Haixin Xu, Seanna Annis, and Frances Trail. Michigan State University, East Lansing, MI.

E-5 **"Repression of aflatoxin biosynthetic genes of *Aspergillus flavus* by acetosyringone."** Sui-Sheng T. Hua,[1] B.-H. Liu,[1] G. A. Payne,[2] F. S. Chu,[3] M. Flores-Espiritu,[1] J. L. Baker.[1] [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2] North Carolina State University, Raleigh, NC; [3]University of Wisconsin, Madison, WI.

Poster Board Number

E-6 **"Resistance of almond and walnut varieties to aflatoxin production."** Noreen Mahoney,[1] Russell J. Molyneux,[1] Bruce C. Campbell,[1] Gale McGranahan,[2] and Thomas M. Gradziel.[2] [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2]University of California, Davis, CA.

E-7 **"Effects of naphthoquinones from walnuts on aflatoxin production and germination of *Aspergillus flavus*."** Noreen Mahoney,[1] Russell J. Molyneux,[1] Bruce C. Campbell,[1] and Gale McGranahan.[2] [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2]University of California, Davis, CA.

E-8 **"Volatiles of walnut hybrids and species by headspace GCMS analysis: A potential aid in selecting varieties resistant to disease and insect attack."** James Roitman,[1] Bruce C. Campbell,[1] Glory Merrill,[1] and Thierry Bourgoin.[2] [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2]Laboratorie d'Entomologie, Museum National d'Histoire Naturelle, Paris, France.

E-9 **"Volatiles from some yeasts grown in vitro inhibit aflatoxin production by *Aspergillus flavus* -- GCMS analysis of headspace volatiles of isolate K-1."** James N. Roitman, Sui-Sheng Hua, James Baker, and Bruce Campbell. USDA, ARS, Western Regional Research Center, Albany, CA.

E-10 **"Timing of aflatoxin biosynthesis, triglyceride hydrolysis and gluconeogenesis in a medium simulating cottonseed."** Jay E. Mellon, Peter J. Cotty, and Michael K. Dowd. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

E-11 **"Isolation of a new chitinase from corn line Tex6."** Kenneth G. Moore,[1] Donald G. White,[2] and Gary A. Payne.[1] [1]North Carolina State University, Raleigh, NC; [2]University of Illinois, Champaign, IL.

E-12 **"Potential use of native plant compounds for the prevention of aflatoxin contamination."** Douglas M. Light, Katherine M. Reynolds, Ronald G. Buttery, Gloria Merrill, James N. Roitman, and Bruce C. Campbell. USDA, ARS, Western Regional Research Center, Albany, CA.

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